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(54) **Title:** BIOMARKERS FOR NAD(+)-DIPHTHAMIDE ADP RIBOSYLTRANSFERASE RESISTANCE

(57) **Abstract:** Phosphorylation of the serine residue at position 615 of eukaryotic elongation factor 2 (eEF2) protein is associated with resistance to NAD(+)-diphthamide ADP-ribosyltransferase enzymes such as *Pseudomonas* exotoxin, diphtheria toxin and cholera toxin, which are used in targeted immunotherapies. Methods of determining resistance and related treatments are provided.



BIOMARKERS FOR NAD(+)-DIPHTHAMIDE ADP RIBOSYLTRANSFERASE RESISTANCEField of the invention

The invention relates to methods of assessing resistance to treatment with
5 NAD(+)-diphthamide ADP ribosyltransferases, for example *Pseudomonas*
exotoxin A (PE), and to related methods of treatment and medical uses.

Background

Pseudomonas exotoxin A (PE) is a bacterial toxin with cytotoxic activity
10 against eukaryotic cells. As such, truncated variants of PE lacking cell
binding properties are being used for destroying or inhibiting the growth
of undesired cells, such as cancer cells. Generally, truncated variants of
PE are linked to a targeting agent, such as an antibody, to direct the
toxin to target cells while sparing other cells.

15 The mechanism of action of PE is to inactivate eukaryotic elongation factor
2 (eEF2), thereby inhibiting protein translation and leading to cell death.
Inactivation occurs by ADP-ribosylation of a diphthamide residue of eEF2.
The diphthamide residue is produced by post-translational modification of a
20 histidine residue at position 715 of the human eEF2 sequence.

Other bacterial toxins share the same mechanism of action, including
diphtheria toxin (DT) and cholix toxin from *Vibrio cholerae* (Jørgensen et
al. 2008a). These toxins are accordingly classified as NAD(+)-diphthamide
25 ADP ribosyltransferase enzymes (EC 2.4.2.36). (Note, cholix toxin is
distinct from cholera toxin, which ADP-ribosylates an arginine residue of
the GTP-binding protein G_s.)

Summary of the invention

30 In the work underlying the present invention, the inventors have found that
serine phosphorylation of human eEF2 is associated with resistance to ADP-
ribosylation of eEF2 by PE.

Human eEF2 contains two potential serine phosphorylation sites, at
35 positions 502 and 595 (hereafter "S502" and "S595", respectively).
However, it is known that serine phosphorylation of the eEF2 molecule
occurs predominantly at the S595 position (Hizli et al. 2013). Moreover,
the present inventors deduce that the S595 residue lies in very close
proximity to the diphthamide residue at position 715 in the three-
40 dimensional structure of the eEF2 protein, and that phosphorylation of the
S595 induces a local conformational change that is consistent with

inhibition of ADP-ribosylation of the diphthamide residue at position 715. Furthermore, the present inventors show that ADP-ribosylation of the diphthamide residue at position 715 interferes with serine phosphorylation of eEF2. Taking all these points together, the inventors conclude that phosphorylation of the S595 residue is associated with resistance to ADP-ribosylation of eEF2 by PE.

However, in accordance with the work underlying the invention, the inventors consider that non-site-specific serine phosphorylation of eEF2 is also indicative of resistance to ADP-ribosylation of eEF2 by PE. Because serine phosphorylation at the S595 site predominates the overall level of serine phosphorylation of the eEF2 protein, non-site-specific serine phosphorylation of eEF2 may therefore represent a proxy for eEF2 phosphorylation at the S595 residue.

The inventors also propose that the findings with PE will apply also to other NAD(+)-diphthamide ADP ribosyltransferase enzymes having the same mechanism of action as PE.

Accordingly, in a first aspect, the invention provides a method for assessing resistance or non-resistance of diseased cells in a patient to treatment with a NAD(+)-diphthamide ADP-ribosyltransferase, the method comprising assaying for serine phosphorylation of eEF2 protein in a sample containing the diseased cells, wherein serine phosphorylation of the eEF2 protein is indicative that the diseased cells are resistant to treatment with a NAD(+)-diphthamide ADP-ribosyltransferase.

The method may include a step of selecting the patient for treatment with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to diseased cells of the patient if the diseased cells are assessed to be non-resistant to NAD(+)-diphthamide ADP ribosyltransferase.

Additionally or alternatively, the method may include a step of deselecting the patient for treatment with a NAD(+)-diphthamide ADP ribosyltransferase if the diseased cells are assessed to be resistant to NAD(+)-diphthamide ADP ribosyltransferase, or a step of selecting the patient for treatment with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to diseased cells of the patient in combination with an agent that inhibits

phosphorylation of the S595 residue of eEF2 if the diseased cells are assessed to be resistant to NAD(+)-diphthamide ADP ribosyltransferase.

In a related second aspect, the invention provides a method for selecting and/or deselecting a patient for treatment with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to diseased cells of the patient, the method comprising:

- (i) assaying for serine phosphorylation of eEF2 protein in a sample containing diseased cells from the patient; and
- (ii) (a) selecting the patient for treatment with a NAD(+)-ribosyltransferase targeted to diseased cells of the patient if the assay is negative for serine phosphorylation of the eEF2 protein; and/or either
- (ii) (b) (1) deselecting the patient for treatment with a NAD(+)-ribosyltransferase targeted to diseased cells of the patient if the assay is positive for serine phosphorylation of the eEF2 protein; or
- (ii) (b) (2) selecting the patient for treatment with a NAD(+)-ribosyltransferase targeted to diseased cells of the patient in combination with an agent that inhibits phosphorylation of the S595 residue of eEF2 if the assay is positive for serine phosphorylation of the eEF2 protein.

Following the selection of a patient for treatment with the targeted therapeutic agent, the patient may be treated with the targeted therapeutic agent (in combination with an agent that inhibits phosphorylation of the S595 residue of eEF2 in the case where the eEF2 protein is serine phosphorylated).

Accordingly, in a third aspect, the invention provides a method for treating a patient having a condition that is treatable by cytotoxic activity targeted to diseased cells of the patient, the method comprising:

assaying a sample containing diseased cells from a patient for serine phosphorylation of eEF2 protein; and either

treating a patient in whose sample the assay is negative for serine phosphorylation of eEF2 protein with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to diseased cells of the patient; or

treating a patient in whose sample the assay is positive for serine phosphorylation of eEF2 protein with a targeted therapeutic agent

comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to diseased cells of the patient in combination with an agent that inhibits phosphorylation of the S595 residue of eEF2.

- 5 Similarly, the invention provides a method for treating a patient having a condition that is treatable by cytotoxic activity targeted to diseased cells of the patient, the method comprising:
- assaying for serine phosphorylation of eEF2 protein in a sample containing diseased cells from the patient;
- 10 assessing resistance or non-resistance of the diseased cells to treatment with a NAD(+)-diphthamide ADP-ribosyltransferase, wherein serine phosphorylation of the eEF2 protein is indicative that the diseased cells are resistant to treatment with a NAD(+)-diphthamide ADP-ribosyltransferase; and either
- 15 treating a patient whose diseased cells are assessed to be non-resistant with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to diseased cells of the patient; or
- treating a patient whose diseased cells are assessed to be resistant
- 20 with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to diseased cells of the patient in combination with an agent that inhibits phosphorylation of the S595 residue of eEF2.
- 25 Further, the invention provides a method for treating a patient having a condition that is treatable by cytotoxic activity targeted to diseased cells of the patient, the method comprising:
- treating the patient with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent
- 30 targeted to diseased cells of the patient, wherein the patient is selected for treatment with the targeted therapeutic agent on the basis of a negative assay result for serine phosphorylation of eEF2 protein in a sample containing diseased cells from the patient; or
- treating the patient with a targeted therapeutic agent comprising a
- 35 NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to diseased cells of the patient in combination with an agent that inhibits phosphorylation of the S595 residue of eEF2, wherein the patient is selected for treatment with a NAD(+)-diphthamide ADP ribosyltransferase in combination with an agent that inhibits phosphorylation of the S595
- 40 residue of eEF2 on the basis of a positive assay result for serine

phosphorylation of eEF2 protein in a sample containing diseased cells from the patient.

In a fourth aspect, the invention provides a NAD(+)-diphthamide ADP
5 ribosyltransferase for use in a method of medical treatment of a patient from whom a sample containing diseased cells has given a negative result in an assay for serine phosphorylation of eEF2 protein.

The invention also provides a NAD(+)-diphthamide ADP ribosyltransferase for
10 use in a method of medical treatment of a patient from whom a sample containing diseased cells has been assayed for serine phosphorylation of eEF2 protein and assessed as non-resistant to NAD(+)-diphthamide ADP ribosyltransferase treatment.

15 Similarly, the invention provides a NAD(+)-diphthamide ADP ribosyltransferase for use in combination with an agent that inhibits phosphorylation of the S595 residue of eEF2 in a method of medical treatment of a patient from whom a sample containing diseased cells has given a positive result in an assay for serine phosphorylation of eEF2
20 protein.

The invention also provides an agent that inhibits phosphorylation of the S595 residue of eEF2, for use in combination with a NAD(+)-diphthamide ADP ribosyltransferase in a method of medical treatment of a patient from whom
25 a sample containing diseased cells has given a positive result in an assay for serine phosphorylation of eEF2 protein.

Similarly, the invention provides a NAD(+)-diphthamide ADP ribosyltransferase for use in combination with an agent that inhibits
30 phosphorylation of the S595 residue of eEF2 in a method of medical treatment of a patient from whom a sample containing diseased cells has been assayed for serine phosphorylation of eEF2 protein and assessed as resistant to NAD(+)-diphthamide ADP ribosyltransferase treatment.

35 The invention also provides an agent that inhibits phosphorylation of the S595 residue of eEF2, for use in combination with a NAD(+)-diphthamide ADP ribosyltransferase in a method of medical treatment of a patient from whom a sample containing diseased cells has been assayed for serine phosphorylation of eEF2 protein and assessed as resistant to NAD(+)-
40 diphthamide ADP ribosyltransferase treatment.

In this aspect of the invention, the NAD(+)-diphthamide ADP ribosyltransferase is preferably coupled to a cell-binding agent targeted to diseased cells of the patient, as a targeted therapeutic agent.

- 5 The invention also provides a NAD(+)-diphthamide ADP ribosyltransferase for use in any of the methods of treatment otherwise described herein.

10 The invention also provides an agent that inhibits phosphorylation of the S595 residue of eEF2 for use in any of the methods of treatment otherwise described herein.

- 15 In a fifth aspect, the invention provides a pharmaceutical combination comprising a NAD(+)-diphthamide ADP ribosyltransferase and an agent that inhibits phosphorylation of the S595 residue of eEF2. As explained above, such a combination treatment is expected to be useful in the treatment of patients whose diseased cells have given a positive result in an assay for serine phosphorylation of eEF2 protein and/or whose diseased cells have been assayed for serine phosphorylation of eEF2 protein and assessed as resistant to treatment with a NAD(+)-diphthamide ADP ribosyltransferase.
- 20 In addition, however, it is contemplated that such a combination treatment may be used without testing patients for serine phosphorylation of eEF2, in particular to increase the response rate compared to treatment with NAD(+)-diphthamide ADP ribosyltransferase alone.

- 25 Similarly, the invention provides a NAD(+)-diphthamide ADP ribosyltransferase for use in a method of medical treatment of a patient having a condition that is treatable by cytotoxic activity targeted to diseased cells of the patient, wherein the treatment comprises administering the NAD(+)-diphthamide ADP ribosyltransferase in combination
- 30 with an agent that inhibits phosphorylation of the S595 residue of eEF2.

- Likewise, the invention provides an agent that inhibits phosphorylation of the S595 residue of eEF2 for use in a method of medical treatment of a patient having a condition that is treatable by cytotoxic activity targeted
- 35 to diseased cells of the patient, wherein the treatment comprises administering the agent that inhibits phosphorylation of the S595 residue of eEF2 in combination with a NAD(+)-diphthamide ADP ribosyltransferase.

- In this aspect of the invention also, the NAD(+)-diphthamide ADP
- 40 ribosyltransferase is preferably coupled to a cell-binding agent targeted to diseased cells of the patient, as a targeted therapeutic agent.

In all preceding aspects and embodiments of the invention, a NAD(+)-diphthamide ADP ribosyltransferase that is administered to (or that is for administration to) a patient will be coupled to a cell-binding agent targeted against diseased cells of the patient as a targeted therapeutic agent. The NAD(+)-diphthamide ADP ribosyltransferase is preferably coupled to the cell-binding agent as a fusion polypeptide, either directly or indirectly via a linker. In preferred embodiments, the fusion is direct. Coupling may also be by chemical conjugation.

A preferred cell-binding agent is an antibody, in particular an antibody directed against a tumour- or cancer-specific antigen. Exemplary antibodies and antigens are described below.

In all preceding aspects and embodiments of the invention, the NAD(+)-diphthamide ADP ribosyltransferase is preferably a PE toxin, diphtheria toxin or cholix toxin, more preferably a PE toxin or diphtheria toxin, still more preferably a PE toxin. Further preferred forms of PE toxin are described below. These preferences apply independently to the NAD(+)-diphthamide ADP ribosyltransferase that is administered (or that is for administration) to the patient and to the methods of assessing resistance to treatment with a NAD(+)-diphthamide ADP ribosyltransferase. Thus, for example, a method of the invention may involve determining that diseased cells of a patient are resistant to treatment with NAD(+)-diphthamide ADP ribosyltransferases generally, and administering a preferred NAD(+)-diphthamide ADP ribosyltransferase such as a PE toxin.

In all preceding aspects and embodiments of the invention, the patient is preferably a patient having a condition that is treatable by cytotoxic activity targeted to diseased cells of the patient. The condition is preferably a cancer or tumour. However, the invention is not limited to the treatment of cancer and tumour. Other conditions may also be treatable by cytotoxic activity targeted to diseased cells of the patient, including viral infections such as HIV, rabies, EBV and Kaposi's sarcoma-associated herpesvirus, and autoimmune diseases such as multiple sclerosis and graft-versus-host disease drugs.

The inventors have screened more than 200 kinases for the activity of phosphorylating the S595 residue of eEF2, and found that only CDK2, CDK1 and NEK have this activity. Accordingly, in all preceding aspects and embodiments of the invention, the agent that inhibits phosphorylation of the S595 residue of eEF2 is preferably an inhibitor of CDK2, CDK1 or NEK.

An inhibitor of CDK2 and/or CDK1 may be a pan-CDK inhibitor. Exemplary agents are described below.

In all preceding aspects and embodiments of the invention, the patient is preferably a human.

In all preceding aspects and embodiments of the invention in which patients are treated or selected for treatment with a NAD(+)-diphthamide ADP ribosyltransferase on the basis of a negative assay result for serine phosphorylation of eEF2 protein or following an assessment of non-resistance to NAD(+)-diphthamide ADP ribosyltransferase, the treatment may exclude treatment with any agent that is known to inhibit phosphorylation of the S595 residue of eEF2, such as those described herein, or to promote dephosphorylation of the S595 residue of eEF2.

Although a wide variety of assays are available both for non-specific serine phosphorylation of the eEF2 protein and for phosphorylation of the eEF2 protein specifically at the S595 residue, the use of antibody-based assays are preferred for all preceding aspects and embodiments of the invention. Antibodies that selectively bind to eEF2 or a fragment thereof that is phosphorylated at the S595 residue, relative to eEF2 or a fragment thereof that is non-phosphorylated at the S595 residue (hereafter, "eEF2-pS595-specific antibodies"), are particularly preferred. An exemplary eEF2-pS595-specific antibody, referred to as 4F2, is provided in Example 8 herein. 4F2, other eEF2-pS595-specific antibodies derived therefrom, and other antibodies obtained in a similar manner to 4F2 are therefore specifically contemplated for use in the assays of the invention.

Accordingly, in a sixth aspect, the invention further provides a monoclonal anti-eEF2 antibody, wherein the antibody binds to eEF2 that is phosphorylated at the S595 residue with higher binding affinity than to eEF2 that is non-phosphorylated at the S595 residue. The higher binding affinity is preferably at least 10-fold higher, more preferably at least 100-fold higher, more preferably at least 1000-fold higher. More preferably, the antibody substantially does not bind to eEF2 that is non-phosphorylated at the S595 residue.

Preferably the antibody binds to eEF2 that is phosphorylated at the His715 residue with a K_D of 100 nM or less, 10 nM or less, 1 nM or less, 100 pM or less, 10 pM or less, or 1 pM or less.

The antibody may have the heavy chain variable domain sequence of SEQ ID NO:53, or a heavy chain variable domain sequence having at least 80%, 85%, 90%, 95%, 98% or 99% identity to SEQ ID NO:53.

- 5 Additionally or alternatively, the antibody may have the light chain variable domain sequence of SEQ ID NO:54, or a light chain variable domain sequence having at least 80%, 85%, 90%, 95%, 98% or 99% identity to SEQ ID NO:54.
- 10 The antibody may have at least the heavy chain complementarity-determining region (CDR) sequence H3 of SEQ ID NO:60. Preferably the antibody has the heavy chain CDRs H2 of SEQ ID NO:58 or 59 and H3 of SEQ ID NO:60. More preferably the antibody has the heavy chain CDRs H1 of SEQ ID NO:55, 56 or 57, H2 of SEQ ID NO:58 or 59 and H3 of SEQ ID NO:60. All combinations of
- 15 CDRH1 and CDRH2 sequences are specifically envisaged (namely SEQ ID NOs:55 and 58, 55 and 59, 56 and 58, 56 and 59, 57 and 58, and 57 and 59), though SEQ ID NO: 55 and 58 are preferred.
- 20 Additionally or alternatively, the antibody may have at least the light chain complementarity-determining region (CDR) sequence L3 of SEQ ID NO:63). Preferably the antibody has the light chain CDRs L2 and L3 of SEQ ID NOs:62 and 63). More preferably the antibody has the light chain CDRs L1, L2 and L3 of SEQ ID NOs:61, 62 and 63.
- 25 However, it is also contemplated that one or more of the CDR sequences of the 4F2 antibody may be altered to a certain extent without loss of the binding properties described above. Accordingly, the antibody may have the CDR-H1 sequence of SEQ ID NO:55, 56 or 57 (preferably SEQ ID NO:55), or said CDR-H1 sequence with one or more amino acid insertions, deletions
- 30 and/or substitutions. The antibody may have the CDR-H2 sequence of SEQ ID NO:58 or 59 (preferably SEQ ID NO:58), or said CDR-H2 sequence with one or more amino acid insertions, deletions and/or substitutions. The antibody may have the CDR-H3 sequence of SEQ ID NO:60, or said CDR-H3 sequence with one or more amino acid insertions, deletions and/or substitutions. The
- 35 antibody may have the CDR-L1 sequence of SEQ ID NO:61, or said CDR-L1 sequence with one or more amino acid insertions, deletions and/or substitutions. The antibody may have the CDR-L2 sequence of SEQ ID NO:62, or said CDR-L2 sequence with one or more amino acid insertions, deletions and/or substitutions. The antibody may have the CDR-L3 sequence of SEQ ID
- 40 NO:63, or said CDR-L3 sequence with one or more amino acid insertions, deletions and/or substitutions.

The invention further provides a monoclonal antibody comprising the CDR H1-H3 sequences shown in SEQ ID NOs:55 or 56 or 57 (for H1, preferably SEQ ID NO:55), and 58 or 59 (for H2, preferably SEQ ID NO:58) and 60 (for H3).

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The invention further provides a monoclonal antibody comprising the heavy chain variable domain sequence shown in SEQ ID NO:53 and the light chain variable domain sequence shown in SEQ ID NO:54.

10 The antibodies of the invention are preferably labelled with a detectable label, such as an enzyme, a fluorescent label, a radiolabel, an electroluminescent label or biotin.

15 The antibodies of the invention may be used in the methods of the preceding aspects and embodiments of the invention. Further, the invention also provides the use of an antibody of the invention in an *in vitro* method of assessing resistance or non-resistance of a cell population to NAD(+)-diphthamide ADP ribosyltransferases treatment. The descriptions and definitions of suitable and preferred NAD(+)-diphthamide ADP
20 ribosyltransferases provided elsewhere herein apply here also.

Embodiments of the present invention will now be described by way of example and not limitation with reference to the accompanying figures. However various further aspects and embodiments of the present invention
25 will be apparent to those skilled in the art in view of the present disclosure.

"and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. For
30 example "A and/or B" is to be taken as specific disclosure of each of (i) A, (ii) B and (iii) A and B, just as if each is set out individually herein.

Unless context dictates otherwise, the descriptions and definitions of the
35 features set out above are not limited to any particular aspect or embodiment of the invention and apply equally to all aspects and embodiments which are described.

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Description of the figures

Figure 1A shows a structure model of human eEF2. The model was generated by humanizing the X-ray structure PDB:3B82 (complex of *S. cerevisiae* eEF2, PE and NAD⁺). The protein surface that interacts with PE is identical between human and yeast. None of the amino acid changes that were implemented to humanize the yeast-derived structure generated structural incompatibilities, or generated alterations in proximity to the phosphorylation sites at T56 and S595, or alterations close to H715 and its attached diphthamide. A discrepancy in position designation was observed between sequence files and the literature, with T56 actually being in sequence position 57 in the sequence. For unambiguous identification, 'T56' refers to the amino acid that is underlined in the sequence stretch AGETRFTDTR (SEQ ID NO: 5) of eEF2.

Figure 1B shows a structure model of human eEF2 complexed with *Pseudomonas* exotoxin A. The model was generated by humanizing the X-ray structure PDB:3B82 (complex of *S. cerevisiae* eEF2, PE and NAD⁺). The protein surface that interacts with PE is identical between human and yeast. None of the amino acid changes that were implemented to humanize the yeast derived structure generated structural incompatibilities, or generated alterations in proximity to the phosphorylation sites at T56 and S595, or alterations close to H715 and its attached diphthamide.

Figure 1C shows that phospho-serine at position 595 of eEF2 is incompatible with the human eEF2 model and published structures of eEF2. In this inward-facing orientation, S595 is not accessible to kinases and the phosphate would clash with N597 and N600.

Figure 1D shows that trans to cis isomerization of the P596 residue 'flips' the S595-containing loop and thereby directs S595 to the surface of eEF2. The 'flip' was modeled by superimposing a cis-trans peptide 'GP' from the structure 4ICB and subsequently placing pS according to the coordinates of the cis peptide. This alters the orientation of S595 but not the remainder of the structure. In this orientation, S595 is accessible to kinases and phosphorylation is structurally compatible. However, cis-pS595 extends into and hence disturbs the interface of eEF2 and PE.

Figure 2 shows that ADP-ribosylation of eEF2 changes the S595 region of eEF2 and thereby reduces serine phosphorylation. Diphthamide-containing MCF-7 wildtype cells that are exposed to PE become ADP-ribosylated at H715 in close proximity to S595. This in turn reduces the S-phosphorylation at

eEF2. Dph1 ko cells which do not carry a diphthamide and hence cannot be ADP-ribosylated do not show reduced S-phosphorylation upon toxin exposure.

Figure 3A shows the procedure for generation of toxin-resistant DPH-
5 knockout cell lines. MCF-7 cells were transfected with zinc finger nucleases and incubated for 3 days. Then cells were selected with toxin and frequently washed to remove dying cells. Single cell clones were seeded in a 96-well plate without toxin and expanded till further analysis.

10 Figure 3B shows DPH1 gene sequence analysis of MCF-7 DPH1ko cell clones 3 (SEQ ID NOs: 47-49) and 4 (SEQ ID NOs: 50 & 51). Genomic DNA was isolated of each cell line (DPH1ko clone 3 upper panel, DPH1ko clone 4 lower panel) and a fragment containing the zinc finger binding site was amplified by PCR. PCR product was purified and TOPO® TA cloned. Plasmid DNA was
15 isolated from 10 bacterial clones and sequence analysis was performed. Here only two representative sequences are shown. For clone 3 two alleles were found, with two out-of-frame mutations (no wildtype allele remaining). For clone 4, two alleles were identified, with only one out of frame mutation.

20 Figure 3C shows the viability of MCF-7 wt and MCF-7 DPH1ko cell clones 3 and 4 after toxin treatment. MCF-7 cells were treated with toxins PE, DT and cycloheximide (CHX), respectively. After 72 h incubation a cytotoxic assay was performed. DPH1ko cells were absolutely resistant to PE (upper
25 left panel) and DT (upper right panel). There was no sensitivity change between CHX-treated MCF-7 wt and DPH1ko (lower panel).

Fig. 4 shows that diphthamide is required for ADP-ribosylation of eEF2, but not for T56 phosphorylation of eEF2 using toxin-resistant DPH1 knockout
30 cell lines. (a) eEF2 of wildtype MCF-7 cells becomes ADP-ribosylated at diphthamide by PE. eEF2 of MCF-7 dph1ko cells does not have diphthamide and is therefore not ADP-ribosylated by PE. (b) eEF2 can be detected in DPH1ko cells with anti-eEF2 antibodies after phosphoserine immunoprecipitation. This indicates that diphthamide is not needed to phosphorylate eEF2 at
35 serine. (c) NH125 treatment induces T56 phosphorylation in MCF7 wildtype cells as well as in MCF7dph1ko cells, indicating that diphthamide is not necessary for T56 phosphorylation.

Figure 5A shows a principal component analysis (PCA) of the transcriptional
40 responses of MCF-7 cells that were exposed for 7hrs to IC50 concentrations of truncated *Pseudomonas* exotoxin (PE), geldanamycin (GA), cycloheximide

(CHX) or alpha amanitin (AMA). Clear separation of responses is indicated by the PCA for toxins with different mode of action. PE and CHX are more close to each other. Their mechanism of action is also similar since both cause inhibition of translation.

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Figure 5B shows a comparison of genes that are induced 7hrs after exposure of MCF7 cells to IC50 concentrations of truncated *Pseudomonas* exotoxin (PE), geldanamycin (GA), cycloheximide (CHX) or alpha amanitin (AMA). AAR* is the gene set which is upregulated in HepG2 cells that were subjected to amino acid starvation. Note the similarity of the responses of cells exposed to protein synthesis inhibitors (CHX and PE) with AAR even though different cell types were analyzed, and the dissimilarity between protein synthesis inhibitors (CHX and PE) to GA and AMA even though the same cell type was analyzed.

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Figure 6 shows that ADP-ribosylation of eEF2 is more stable than S595 and T56 phosphorylation. (a) Without phosphatase inhibitors, T56 phosphorylation of eEF2 decreases within minutes. Because S-phosphorylation has generally the same instability as T-phosphorylation, decline in pT56 signals can also serve as surrogate to measure the instability of phosphoserine modification of eEF2. (b) ADP-ribosylation remains stable for hours.

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Figure 7 shows the influence of S595 alterations on toxin-mediated ADP-ribosylation of eEF2. (A) Recombinant wild-type or mutated eEF2 containing a C-terminal HA tag was expressed in MCF7 cells, subsequently separated from cellular eEF2 by binding to HA-beads, exposed to toxin and Bio-NAD and thereafter purified and analyzed by Western Blots to evaluate diphthamide ADP-ribosylation. (B) Western blot assays with anti-eEF2 and anti-HA demonstrate presence of recombinant eEF2 in all lanes. Only unmodified eEF2 becomes ADP-ribosylated upon toxin exposure (lower panel). The H715A mutated eEF2 does not become ADP-ribosylated because it does not contain a H715-diphthamide as toxin target. (C) Analysis of the ADP-ribosylation of S595 mutants shows that alteration of the 595 position of eEF2 by replacing the serine with alanine, glutamate or aspartate interferes with the toxin-mediated ASDP ribosylation of eEF2 at H715-diphthamide.

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Figure 8 shows the results of BIACORE analysis of binding of a rabbit monoclonal antibody clone 4F2 to a peptide consisting of residues 583-606 of human eEF2, with and without phosphorylation of the serine residue at

position 595. Key: BL - binding late; SL - stability late; k_d - dissociation rate constant; CL - capture level; MR - molar ratio.

Figure 9 shows the results of a Western blot using clone 4F2 to detect eEF2 in extracts from unstimulated (lane 2) and stimulated (lane 3) MCF-7 cells labelled with PI (propidium iodide).

Detailed description of the invention

Definitions

The "S595" residue of eEF2 refers to a serine residue at a position in an eEF2 sequence that corresponds to position 595 in the human eEF2 sequence represented by NCBI accession number NP-001952 (version 1; GI:4503483) when the two eEF2 sequences are aligned.

The "T56" residue of eEF2 refers to a threonine residue at a position in an eEF2 sequence that corresponds to position 57 in the human eEF2 sequence represented by NCBI accession number NP-001952 (version 1; GI:4503483) when the two eEF2 sequences are aligned. This residue is commonly referred to as T56 in the literature so this designation has been retained, despite the discrepancy in numbering. It may be labelled T57 in some publications. For unambiguous identification, 'T56' refers to the amino acid that is underlined in the sequence stretch AGETRFTDTR (SEQ ID NO: 5) of eEF2.

The "H715" residue of eEF2 refers to a histidine residue at a position in an eEF2 sequence that corresponds to position 715 in the human eEF2 sequence represented by NCBI accession number NP-001952 (version 1; GI:4503483) when the two eEF2 sequences are aligned.

"Affinity" refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (K_d). Affinity can be measured by common methods known in the art, including those described herein. Specific illustrative and exemplary embodiments for measuring binding affinity are described in the following.

In one embodiment, K_d is measured by a radiolabeled antigen binding assay (RIA) performed with the Fab version of an antibody of interest and its antigen as described by the following assay. Solution binding affinity of FABs for antigen is measured by equilibrating Fab with a minimal
5 concentration of (^{125}I)-labelled antigen in the presence of a titration series of unlabeled antigen, then capturing bound antigen with an anti-Fab antibody-coated plate (see, e.g., Chen, Y. et al., J. Mol. Biol. 293 (1999) 865-881). To establish conditions for the assay, MICROTITER® multi-well
10 plates (Thermo Scientific) are coated overnight with 5 µg/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23°C). In a non-adsorbent plate (Nunc #269620), 100 pM or 26 pM [^{125}I]-antigen are mixed with serial
15 dilutions of a Fab of interest (e.g., consistent with assessment of the anti-VEGF antibody, Fab-12, in Presta, L.G. et al., Cancer Res. 57 (1997) 4593-4599). The Fab of interest is then incubated overnight; however, the incubation may continue for a longer period (e.g., about 65 hours) to ensure that equilibrium is reached. Thereafter, the mixtures are transferred to the capture plate for incubation at room temperature (e.g.,
20 for one hour). The solution is then removed and the plate washed eight times with 0.1% polysorbate 20 (TWEEN-20®) in PBS. When the plates have dried, 150 µl/well of scintillant (MICROSCINT-20 TM; Packard) is added, and the plates are counted on a TOPCOUNT TM gamma counter (Packard) for ten minutes. Concentrations of each Fab that give less than or equal to 20% of
25 maximal binding are chosen for use in competitive binding assays.

According to another embodiment, K_d is measured using surface plasmon resonance assays using a BIACORE®-2000 or a BIACORE®-3000 (BIAcore, Inc., Piscataway, NJ) at 25°C with immobilized antigen CM5 chips at ~10 response
30 units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIACORE, Inc.) are activated with N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10 mM sodium acetate, pH 4.8, to 5 µg/ml (~0.2 µM) before injection at a flow rate of 5
35 µl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1 M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% polysorbate 20 (TWEEN-20™) surfactant (PBST) at 25°C at a flow rate of
40 approximately 25 µl/min. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model

(BIAcore® Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant (K_d) is calculated as the ratio k_{off}/k_{on} . See, e.g., Chen, Y. et al., J. Mol. Biol. 293 (1999) 865-881. If the on-rate exceeds $10^6 \text{ M}^{-1} \text{ s}^{-1}$ by the surface plasmon resonance assay above, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25°C of a 20 nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped spectrophotometer (Aviv Instruments) or a 8000-series SLM-AMINCO™ spectrophotometer (ThermoSpectronic) with a stirred cuvette.

In all aspects and embodiments of the invention, unless the context requires otherwise, absolute binding affinity values and relative binding affinities are preferably determined by surface plasmon resonance. In the case of antibodies, binding affinity is preferably determined using the Fab (or other monovalent) form of the antibody.

As will be evident from the foregoing, "higher binding affinity" therefore corresponds to a numerically lower K_d value. Conversely, the term "a K_d of [value] or higher" refers to antibodies with the specified binding affinity or lower binding affinity (higher K_d).

"Substantially does not bind" may refer to a level of binding that is undetectable and/or indistinguishable from non-specific binding by standard techniques for assessing antibody binding, such as western blot, denaturing or non-denaturing gel electrophoresis, immunostaining or ELISA. Additionally or alternatively, it may refer to a K_d of about 1 μM or higher, preferably about 10 μM or higher, about 100 μM or higher or about 1 mM or higher.

The term "combination" in the context of medical treatments is used herein to refer to the use of plural pharmaceutical agents in the treatment of the same individual patient. The treatments may be simultaneous, meaning that the pharmaceutical agents are administered at essentially the same time (such as during the same medical appointment); or sequential, meaning that one agent is administered significantly before the other (such as in separate medical appointments). Typically, the pharmaceutical agents will be administered in concurrent treatment cycles, which may be essentially

synchronous, staggered, or involve different treatment intervals for the two pharmaceutical agents.

An "effective amount" of an agent, e.g., a pharmaceutical formulation,

5 refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result. The treatment methods of the invention will use effective amounts of the specified agents.

10 A "patient" is a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates such as monkeys), rabbits, and rodents (e.g., mice and rats). In all aspects and embodiments of the invention, the patient is preferably a human.

15 "Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and
20 introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such
25 as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence
30 comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc.,
35 South San Francisco, California, or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

40 In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid

sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

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$$100 \text{ times the fraction } X/Y$$

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

As used herein, "treatment" (and grammatical variations thereof such as "treat" or "treating") refers to clinical intervention in an attempt to alter the natural course of the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies of the invention are used to delay development of a disease or to slow the progression of a disease.

The term "cancer" as used herein include both solid and haematologic cancers, such as lymphomas, lymphocytic leukemias, lung cancer, non small cell lung (NSCL) cancer, bronchioloalviolar cell lung cancer, bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, gastric cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer,

- cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, mesothelioma, hepatocellular cancer, biliary cancer, neoplasms of the central nervous system (CNS), spinal axis tumours, brain stem glioma, glioblastoma multiforme,
- 5 astrocytomas, schwannomas, ependymomas, medulloblastomas, meningiomas, squamous cell carcinomas, pituitary adenoma and Ewings sarcoma, including refractory versions of any of the above cancers, or a combination of one or more of the above cancers.
- 10 The term "autoimmune disease" includes rheumatologic disorders (such as, for example, rheumatoid arthritis, Sjögren's syndrome, scleroderma, lupus such as SLE and lupus nephritis, polymyositis/dermatomyositis, cryoglobulinemia, anti-phospholipid antibody syndrome, and psoriatic arthritis), osteoarthritis, autoimmune gastrointestinal and liver disorders
- 15 (such as, for example, inflammatory bowel diseases (e.g. ulcerative colitis and Crohn's disease), autoimmune gastritis and pernicious anemia, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, and celiac disease), vasculitis (such as, for example, ANCA-associated vasculitis, including Churg-Strauss vasculitis, Wegener's
- 20 granulomatosis, and polyarteriitis), autoimmune neurological disorders (such as, for example, multiple sclerosis, opsoclonus myoclonus syndrome, myasthenia gravis, neuromyelitis optica, Parkinson's disease, Alzheimer's disease, and autoimmune polyneuropathies), renal disorders (such as, for example, glomerulonephritis, Goodpasture's syndrome, and Berger's disease),
- 25 autoimmune dermatologic disorders (such as, for example, psoriasis, urticaria, hives, pemphigus vulgaris, bullous pemphigoid, and cutaneous lupus erythematosus), hematologic disorders (such as, for example, thrombocytopenic purpura, thrombotic thrombocytopenic purpura, post-transfusion purpura, and autoimmune hemolytic anemia), atherosclerosis,
- 30 uveitis, autoimmune hearing diseases (such as, for example, inner ear disease and hearing loss), Behcet's disease, Raynaud's syndrome, organ transplant, and autoimmune endocrine disorders (such as, for example, diabetic-related autoimmune diseases such as insulin-dependent diabetes mellitus (IDDM), Addison's disease, and autoimmune thyroid disease (e.g.
- 35 Graves' disease and thyroiditis)). More preferred such diseases include, for example, rheumatoid arthritis, ulcerative colitis, ANCA-associated vasculitis, lupus, multiple sclerosis, Sjögren's syndrome, Graves' disease, IDDM, pernicious anemia, thyroiditis, and glomerulonephritis.
- 40 The terms "treat" and "prevent" as well as words stemming therefrom, as used herein, do not necessarily imply 100% or complete treatment or

prevention. Rather, there are varying degrees of treatment or prevention of which one of ordinary skill in the art recognizes as having a potential benefit or therapeutic effect. In this respect, the treatment aspects invention can provide any amount of any level of treatment or prevention of disease (such as cancer) in a mammal. Furthermore, the invention can include treatment or prevention of one or more conditions or symptoms of the disease, e.g., cancer, being treated or prevented. Also, for purposes herein, "prevention" can encompass delaying the onset of the disease, or a symptom or condition thereof.

The term "immunotoxin" is used herein to refer to a composition comprising an antibody or antigen-binding fragment thereof, coupled to a toxic moiety. An alternative term for certain immunotoxins that are generated by genetic fusion of protein components is "cytolytic fusion protein (cFP)". While immunotoxins represent a preferred class of targeted therapeutic agents of the present invention, the targeted therapeutic agents may comprise alternative cell-binding agents as described herein. The applicability of the present invention is therefore not limited to immunotoxins.

In the context of CDRs, the term "one or more amino acid substitutions, deletions and/or insertions" preferably refers to the substitution, deletion and/or insertion of up to 5 amino acids in any CDR, more preferably up to 4 amino acids, more preferably up to 3 amino acids, more preferably 1 or 2 amino acids, more preferably a single amino acid. In the case of SEQ ID NOs:59 and 60 (which are 9 amino acids in length), "one or more" preferably means up to 4 amino acids, more preferably up to 3 amino acids, more preferably 1 or 2 amino acids, more preferably a single amino acid; in the case of SEQ ID NOs:56 and 62 (which are 6 and 7 amino acids in length, respectively), "one or more" preferably means up to 3 amino acids, more preferably 1 or 2 amino acids, more preferably a single amino acid; in the case of SEQ ID NO: 57 (which is 5 amino acids in length), "one or more" preferably means up to 2 amino acids, more preferably a single amino acid.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, combination therapy, contraindications and/or warnings concerning the use of such therapeutic products.

The term "pharmaceutical combination" is used herein to refer to a product that comprises plural (herein typically two) different pharmaceutical agents, which are intended to be used in combination. They may be formulated together or separately, but will be packaged together, typically with a package insert bearing instructions for the use of the pharmaceutical agents in combination.

The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

Assaying for serine phosphorylation of eEF2

The invention includes methods that comprise assaying for serine phosphorylation of eEF2 protein. Preferably these methods comprise assaying for phosphorylation of the S595 residue of eEF2, wherein phosphorylation of the S595 residue is indicative of resistance to treatment with a NAD(+)-diphthamide ADP-ribosyltransferase. Accordingly, in the patient selection methods of the invention, the patient is preferably selected for treatment with NAD(+)-diphthamide ADP ribosyltransferase if the assay is negative for phosphorylation at the S595 residue.

The methods of the invention may involve determining qualitatively the presence or absence of serine phosphorylation of eEF2, wherein the presence of phosphorylation is indicative of resistance to treatment with a NAD(+)-diphthamide ADP ribosyltransferase and the absence of phosphorylation is indicative of non-resistance to treatment with a NAD(+)-diphthamide ADP ribosyltransferase. That is, the diseased cells may be assessed to be resistant to treatment with NAD(+)-diphthamide ADP ribosyltransferase when phosphorylation is present and non-resistant when phosphorylation is absent. Alternatively, the methods may involve determining the extent of phosphorylation, wherein a greater extent of phosphorylation is indicative

of a greater level of resistance to treatment with a NAD(+)-diphthamide ADP ribosyltransferase.

The extent of phosphorylation may be compared to a reference value, wherein the diseased cells are assessed to be resistant to treatment with a NAD(+)-diphthamide ADP ribosyltransferase if the extent of phosphorylation is greater than the reference value and non-resistant to treatment with a NAD(+)-diphthamide ADP ribosyltransferase if the extent of phosphorylation is less than the reference value.

Thus, it will be understood that a negative assay result and/or an assessment that the diseased cells are non-resistant is not necessarily absolute: diseased cells may be assayed as negative for eEF2 phosphorylation and/or assessed to be non-resistant if the eEF2 protein is partially phosphorylated, in particular if the extent of phosphorylation is below a reference value. Likewise, a patient may be selected for treatment with a NAD(+)-diphthamide ADP ribosyltransferase if the eEF2 protein is partially phosphorylated, in particular if the extent of phosphorylation is below a reference value.

The extent of phosphorylation at a given residue, for example at S595 of eEF2, can be quantified by different methods which are known to experts in the field. For example, mass spectrometry technologies or antibody based technologies that specifically detect the position with and without phosphorylation can be applied to determine the relative ratios of phosphorylated and unphosphorylated molecules.

In the foregoing methods of the invention, the means for assaying serine phosphorylation of eEF2 also are not particularly limited. Any method of assaying serine phosphorylation may be used in the practice of the invention. For example, serine phosphorylation of the eEF2 protein may be assayed non-specifically using anti-phosphoserine antibodies (hereafter, "anti-pS antibodies"), which bind selectively to proteins containing phosphorylated serine residues, relative to proteins that lack phosphorylated serine residues. Anti-phosphoserine antibodies are widely available commercially. As explained above, because phosphorylation at residue S595 predominates serine phosphorylation of the eEF2 protein, non-specific serine phosphorylation may be considered as a proxy for S595 phosphorylation. In the case of detection of serine phosphorylation non-specifically, the assays will generally include a prior step of isolating

eEF2 from the sample. This may include immunoprecipitation with anti-eEF2 antibodies, followed by labelling with anti-pS antibodies.

5 eEF2-pS595-specific antibodies (that is, antibodies that selectively bind to eEF2 or a fragment thereof that is phosphorylated at the S595 residue relative to eEF2 or a fragment thereof that is non-phosphorylated at the S595 residue) can be produced according to routine techniques. For example, as described in Example 8, monoclonal antibodies may be obtained from animals that have been immunised with a peptide that contains the S595 residue and is phosphorylated at that residue. The antibodies may then be
10 screened against the phosphopeptide and counterscreened against the corresponding non-phosphorylated peptide. The antibodies may then be tested for the ability to bind to phosphorylated but not non-phosphorylated eEF2 protein. Clone 4F2 obtained in Example 8 is an exemplary antibody
15 obtained using this protocol. Alternatively, antibody libraries such as phage-display libraries may be screened against the phosphopeptide and counterscreened against the non-phosphorylated peptide and the resultant clones tested for the ability to bind to phosphorylated but not non-phosphorylated eEF2 protein. It will be understood that complete
20 selectivity of the antibody for S595 phosphorylation is not essential to the practice of the invention, provided that the assay is able to discriminate the presence of some eEF2 that is phosphorylated at this position. Preferably, however, an eEF2-pS595-specific antibody will have at least 10-fold higher affinity for eEF2 or a fragment thereof that is
25 phosphorylated at the S595 residue than for eEF2 or a fragment thereof that is phosphorylated at the S595 residue, more preferably at least 100-fold higher affinity.

30 Analogous methods may be used to obtain antibodies that selectively bind to eEF2 that is non-phosphorylated at the S595 residue. Such antibodies may be used in methods that involve determining the extent of phosphorylation at the S595 residue.

35 A wide variety of assay techniques using eEF2-pS595-specific antibodies may be used in the practice of the invention, such as ELISA, FACS or IHC. eEF2-pS595-specific antibodies may be labelled for use in such assays, for example with fluorescent or radioactive labels, according to routine techniques.

Brumbaugh et al. (2011), which is incorporated herein by reference, describes techniques for producing phosphosite-specific antibodies, and discusses their use in arrays, flow cytometry and imaging.

- 5 Alternatively, site-specific phosphorylation of eEF2 at position S595 may be assayed by electrophoretic techniques, such as the method of Hizli et al. (2013), following Welcker et al. (2003). Briefly, Hizli et al. used tryptic digestion of eEF2 that had been phosphorylated with ^{32}P -labelled ATP, followed by 2D electrophoresis and visualisation of ^{32}P , to identify
10 which of the predicted tryptic peptides bearing possible phosphorylation sites had been phosphorylated. To determine the extent of phosphorylation at position S595 *in vivo*, they determined the extent to which (unlabelled) eEF2 isolated from cells could be further phosphorylated by cyclin A-CDK2 *in vitro* with ^{32}P -labelled ATP, since cyclin A-CDK2 phosphorylation *in vitro* occurs almost exclusively at S595. The greater the extent of ^{32}P
15 labelling, the greater the *in vitro* phosphorylation (almost exclusively at position S595) and hence the less the eEF2 had been phosphorylated at S595 *in vivo*.
- 20 It is also possible to measure protein phosphorylation using mass-spectrometry techniques such as ESI-TOF, MALDI-TOF and SELDI-TOF, which differentiate phosphorylated from non-phosphorylated peptides. For application of these techniques, it is important to prevent de-phosphorylation during sample preparation prior to mass-spectrometry
25 determination. This can for example be achieved by addition of phosphatase inhibitors early in the sample processing procedures, by inactivation of enzymes with denaturing agents or by cooling, or combinations thereof.

Assays involving the use of eEF2-pS595-specific antibodies are preferred.

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Patient samples

- In the methods of the invention, the sample containing diseased cells is not particularly limited. In preferred embodiments, the sample is a pre-
35 cancer, cancer or tumour sample. In the case of solid pre-cancers, cancers and tumours, the sample may be from a biopsy, or a sample taken following surgical removal of the pre-cancer, cancer or tumour. In the case of haematological pre-cancers and cancers such as leukaemias and lymphomas, the sample may be a blood sample containing pre-cancerous or cancerous
40 blood cells. However, NAD(+)-diphthamide ADP-ribosyltransferases may also be used for the treatment of other conditions in which the destruction of

diseased cells is desired, such as viral infections and autoimmune diseases. So the applicability of the invention is not limited to pre-cancers, cancers and tumours. The sample may contain virally infected cells or autoimmune effector cells, such as autoimmune T-cells or
5 autoantibody-expressing B-cells.

Protein phosphorylation can be unstable, so following removal from the patient's body, the sample may be treated to stabilise eEF2 phosphorylation until the sample is assayed. For example, WO 2010/019952 reports that the
10 level of phosphorylation of ErbB3 and pAKT in lysates from frozen tumour samples decreased as the time elapsed between tumour resection and freezing increased. A noticeable decrease in phosphorylation level occurred even when the tumour sample was frozen within 10 minutes of resection. 20-40% reduction in phosphorylation levels was observed in the 30 minute samples.
15 Accordingly, treatment to stabilise eEF2 phosphorylation is preferably done as soon as possible after removal of the sample from the patient's body, preferably within 30 minutes, more preferably within 10 minutes, more preferably immediately after removal. Alternatively, the sample may be assayed as soon as possible after removal from the patient's body, again
20 preferably within 30 minutes, more preferably within 10 minutes, more preferably immediately after removal.

Preferably, the treatment to stabilise eEF2 phosphorylation is by freezing, which is preferably done rapidly, for example using liquid nitrogen.
25 Alternatively, the sample may be fixed, for example using formalin.

Additionally or alternatively, the treatment to stabilise eEF2 phosphorylation may include treatment of the sample with one or more phosphatase inhibitors, preferably a broad-specificity phosphatase
30 inhibitor cocktail, such as Sigma-Aldrich's Phosphatase Inhibitor Cocktail 3 (Sigma catalogue number P0044), which contains the following individual components with specific inhibitory properties: cantharidin (Sigma-Aldrich catalogue number C7632), which inhibits protein phosphatase 2A; (-)-p-bromolevamisole oxalate (Sigma-Aldrich catalogue number 190047), which
35 inhibits L-isoforms of alkaline phosphatases; and calyculin A (Sigma-Aldrich catalogue number C5552), which inhibits protein phosphatases 1 and 2A.

Treatments

Likewise, insofar as the invention relates to treatment methods and to products for use in methods of treatment, it is applicable to any condition that is treatable by cytotoxic activity targeted to diseased cells of the patient. The treatment is preferably of a tumour or cancer. However, the applicability of the invention is not limited to tumours and cancers. For example, the treatment may also be of viral infection. Immunotoxins directed against viral antigens expressed on the surface of infected cells have been investigated for a variety of viral infections such as HIV, rabies and EBV. Cai and Berger 2011 Antiviral Research 90(3):143-50 used an immunotoxin containing PE38 for targeted killing of cells infected with Kaposi's sarcoma-associated herpesvirus. In addition, Resimmune® (A-dmDT390-bisFv(UCHT1), described below) selectively kills human malignant T cells and transiently depletes normal T cell and is considered to have potential for the treatment of T-cell driven autoimmune diseases such as multiple sclerosis and graft-versus-host disease, as well as T cell blood cancers for which it is undergoing clinical trials. Thus, the applicability of NAD(+)-diphthamide ADP ribosyltransferase treatment is limited only by the availability of suitable targeting moieties.

NAD(+)-diphthamide ADP ribosyltransferases

As explained above, three NAD(+)-diphthamide ADP ribosyltransferases are known to occur naturally as bacterial virulence factors, namely PE, DT and cholix toxin. Of these, cholix toxin was identified most recently (Jørgensen 2008a). It was characterised as a NAD(+)-diphthamide ADP ribosyltransferase by primary sequence identity to PE (32% identity), the presence of a furin protease site for cellular activation, the presence of a C-terminal KDEL (SEQ ID NO: 9) sequence that is presumed to direct the toxin to the endoplasmic reticulum (though this property is not shared by DT) and three signature regions that characterise the catalytic domain of diphthamide-specific toxins according to Yates et al. 2006. In particular, Yates 2006 reports the identification of several putative NAD(+)-diphthamide ADP ribosyltransferases based on a sequence-based search pattern described in Box I of Yates 2006. These putative NAD(+)-diphthamide ADP ribosyltransferases included cholix toxin, which was subsequently verified in Jørgensen 2008a. Other candidate NAD(+)-diphthamide ADP ribosyltransferases may be identified by similar criteria, followed by verification of diphthamide-specific activity, for example following the technique of Example 3 herein.

Further NAD(+)-diphthamide ADP ribosyltransferase enzymes suitable for use in accordance with the present invention may be identified by subjecting the eEF2 to ADP-ribosylation reactions described herein (e.g. utilizing biotinylated-NAD) or similar assays, and monitoring for the production of ADP-ribosylated eEF2. Protein preparations or fractions, bacterial extracts, plant extracts or cell extracts may be applied as the candidate 'toxin-source'. Positive controls for such reactions include PE or DT or Cholix toxin. Negative controls to differentiate from non-specific labelling of eEF2 by the 'toxin sources' include eEF2 variants that do not contain a diphthamide.

Nevertheless, in all aspects and embodiments of the invention, the NAD(+)-diphthamide ADP ribosyltransferase is preferably a PE, DT or cholix toxin.

For the avoidance of doubt, the terms 'PE', 'DT' and 'cholix toxin' are not limited to the naturally occurring protein sequences, but also include recombinant toxins derived from the naturally occurring toxins that retain NAD(+)-diphthamide ADP ribosyltransferase activity. Indeed, the full-length naturally occurring sequences are generally not preferred.

The native PE, DT and cholix toxin proteins belong to the A-B class of cytotoxic proteins, which consist of a cell-binding subunit (B subunit) and a subunit with cytotoxic activity (A subunit). The B subunits of PE and DT in particular have different cell surface targets, but the A subunit of all three proteins has the NAD(+)-diphthamide ADP ribosyltransferase toxic activity. In the case of PE, the cell surface target is the low density lipoprotein receptor related protein (LRP1; also known as CD91 or the $\alpha 2$ -macroglobulin receptor) or the closely related variant LRP1B (Kounnas et al. 1992 J Biol Chem. 267:12420-12423; Pastrana et al. 2005 Biochim Biophys Acta. 1741:234-239). Cholix toxin may have the same cellular targets. In the case of DT, the cell surface target is a membrane-anchored form of the heparin-binding EGF-like growth factor (HB-EGF precursor) (Naglich et al. (1992) Cell 69:1051-61).

PE, DT and cholix toxin are taken up into cells by receptor-mediated endocytosis, and are processed by furin cleavage and reduction of disulphide linkage to activate and release the cytotoxic A subunit.

Both PE and DT have been modified for targeted therapy by removing the B subunits and replacing them with other cell-targeting agents having desired target cell specificity, such as antibodies. For example, A-dmDT390-

bisFv(UCHT1) (Resimmune®) is composed of the first 390 amino acid residues of DT (containing the catalytic domain and translocation domain that translocates the catalytic domain into the cytosol) coupled to two tandem sFv molecules derived from the anti-CD3ε antibody UCHT1. DT has also been coupled to IL-2 as denileukin difitox for targeting to cells bearing IL-2 receptors in the treatment of leukaemias and lymphomas. Numerous antibodies have been coupled to truncated forms of PE for a variety of therapeutic purposes, as detailed below. In the practice of the present invention, forms of NAD(+)-diphthamide ADP ribosyltransferase which lack a functional native receptor-binding portion are greatly preferred.

Pseudomonas exotoxins (PEs)

Native, wild-type *Pseudomonas* exotoxin A is a 66kD bacterial toxin secreted by *Pseudomonas aeruginosa*, having the 613 amino acid sequence shown in SEQ ID NO:1 and also disclosed in US 5,602,095. This sequence is shown without the native signal peptide, which is shown as the first 25 amino acids of UniProt accession number P11439.2 (gi: 12231043).

The native protein has three major structural domains. The N-terminal domain I comprises two subdomains Ia (amino acids 1-252) and Ib (amino acids 365-399) that are structurally adjacent but separated in the primary amino acid sequence.

Domain I and in particular domain Ia is the cell-binding domain. The function of domain Ib remains undefined. Domain I forms the major component of the B subunit. In the practice of the present invention, forms of PE in which the native domain Ia sequence is omitted or disrupted, and which consequently are unable to bind to LRP1 or LRP1B, are greatly preferred.

Domain II (amino acids 253-364) has been reported to mediate translocation into the cytosol, but this remains controversial (Weldon & Pastan 2011).

Domain III (amino acids 400-613) mediates ADP ribosylation of elongation factor 2. The structural boundary between domain Ib and domain III is not fully settled. According to WO2013/040141 it lies between residues 399 and 400, but Weldon and Pastan 2011 place it between residues 404 and 405. However, full catalytic activity requires a portion of domain Ib as well as domain III. Accordingly, the functional domain III of the native toxin is defined to start at residue 395. Amino acids 602-613 have been found to be

inessential for NAD(+)-ribosyltransferase activity, but amino acids 609-613 of the native sequence are required for cytotoxic activity. These form an endoplasmic reticulum localisation sequence (WO 91/09948, Chaudhary et al 1991, Seetharam et al. 1991). Cytotoxicity can be maintained or enhanced
5 by replacing the native ER localisation sequence with one or more other ER localisation sequences. Accordingly the functional domain III of native PE is considered to consist of residues 395-601.

An extensive body of work has been published disclosing variants and
10 improvements of the native PE molecule for use in targeted cytotoxins. The terms "*Pseudomonas* exotoxin A", "*Pseudomonas* exotoxin" and "PE" as used herein are intended to encompass these and other variants and improvements of native PE that retain cytotoxic activity. In particular, the terms
15 "*Pseudomonas* exotoxin A", "*Pseudomonas* exotoxin" and "PE" are specifically intended to include the variants and improvements disclosed in WO88/02401A1, WO90/12592A1, WO91/09949, WO91/09965, WO93/25690, WO97/13529, WO98/20135, WO2005/052006, WO2007/016150, WO2007/031741, WO2009/32954, WO2011/32022, WO2012/154530, WO2012/170617, WO2013/40141, Mazor R, et al PNAS 111 (2014) 8571-8576, Alewine C, et al, Mol Cancer Ther. (2014) 2653-
20 61 and WO2015/51199. All these publications are incorporated herein in their entirety for the purpose of exemplifying variants and improvements of *Pseudomonas* exotoxin A / PE that are suitable for use in the present invention and that are included, without limitation, within the terms "*Pseudomonas* exotoxin A", "*Pseudomonas* exotoxin" and "PE", with those
25 variants and improvements disclosed in WO2005/052006, WO2007/016150, WO2007/031741, WO2009/32954, WO2011/32022, WO2012/154530, WO2012/170617, and WO2013/40141 being preferred and those disclosed in WO2009/32954, WO2011/32022, WO2012/154530, WO2012/170617, WO2013/40141, Mazor R, et al PNAS 111 (2014) 8571-8576, Alewine C, et al, Mol Cancer Ther. (2014) 2653-
30 61 and WO2015/051199 being particularly preferred.

It is anticipated that further variants and improvements of PE will be developed in future. Since the present invention relates to resistance against the cytotoxic effects of PE, it is anticipated that any such future
35 variants and improvements of PE that retain cytotoxic activity may also be used in the practice of the invention and are therefore included within the terms "*Pseudomonas* exotoxin A" and "PE".

Generally, a PE toxin will have a polypeptide sequence comprising a PE
40 functional domain III having at least 50% amino acid sequence identity over the full length of residues 395-601 of SEQ ID NO:1, wherein the PE toxin

has cytotoxic activity when introduced into a eukaryotic (preferably mammalian) cell. Preferred forms of PE comprise (1) a PE functional domain III having at least 50% amino acid sequence identity over the full length of residues 395-601 of SEQ ID NO:1 and having NAD(+)-diphthamide ADP
5 ribosyltransferase activity, and (2) at least one endoplasmic reticulum localisation sequence. In embodiments in which the PE is coupled to a cell-binding agent as a fusion polypeptide, the PE preferably also comprises (3) a cleavable linker sequence such as a furin-cleavable sequence (FCS) that permits cleavage of the PE functional domain III from
10 the cell-binding agent following uptake into the target cell.

The cleavable linker (such as an FCS) will generally be on the N-terminal side of the PE functional domain III.

15 Other cleavable linkers may be used provided that they permit cleavage of the PE from the cell-binding agent following uptake into the target cell. Furthermore, other means of coupling the PE to the cell-binding agent are contemplated, provided again that they permit separation of the PE from the cell-binding agent following uptake into the target cell. For example, the
20 cell-binding agent may be non-covalently linked to the PE, or linked by disulfide bonds which permit release of the PE moiety under reducing conditions, or linked by other conjugation chemistries that are known in the field of immunoconjugate production.

25 The PE for use in accordance with the present invention will generally lack a functional cell-binding domain I.

Much of the work on PE has focussed on eliminating portions of the native sequence that are unnecessary and/or disadvantageous for use in targeted
30 therapies. For example, replacement of the B (receptor-binding) subunit with another cell-binding agent has reduced the non-specific toxicity of the molecule. Further removal of inessential sequences has reduced immunogenicity. This has led in particular to the development of the following truncated forms of PE: PE40, PE35, PE38, PE38QQR, PE-LR and PE24.
35 PE40 is a truncated derivative of PE (Pai et al 1991 Proc. Natl. Acad. Sci. USA 88:3358-62 and Kondo et al. 1988 J. Biol. Chem. 263:9470-9475). PE35 is a 35 kD carboxyl-terminal fragment of PE in which amino acid residues 1-279 have been deleted and the molecule commences with a Met at position 280 followed by amino acids 281-364 and 381-613 of PE as defined by reference
40 to SEQ ID NO:1. PE35 and PE40 are disclosed, for example, in US 5,602,095,

US 4,892,827, WO93/25690 and WO88/02401, each of which is incorporated herein by reference in its entirety.

5 PE38 contains the translocating and ADP ribosylating domains of PE but not the cell-binding portion (Hwang et al. 1987 Cell 48:129-136). PE38 (SEQ ID NO:2) is a truncated PE pro-protein composed of amino acids 253-364 and 381-613 of SEQ ID NO: 1 which is activated to its cytotoxic form upon processing within a cell (see US 5,608,039, which is incorporated by reference in its entirety herein, and Pastan et al. 1997 Biochim. Biophys. 10 Acta, 1333:C1-C6). PE38QR is a variant of PE38 having mutations of the lysines at positions 590, 606 and 613 of domain III, to permit conjugation to antibodies.

15 PE-LR contains a deletion of domain II except for a furin-cleavable sequence (FCS) corresponding to amino acid residues 274-284 of SEQ ID NO: 1 (RHRQPRGWEQL (SEQ ID NO: 6)) and a deletion of amino acid residues 365-394 of domain Ib. Thus, PE-LR contains amino acid residues 274-284 and 395-613 of SEQ ID NO: 1. PE-LR is described in WO 2009/032954 and Weldon et al 2009, which are each incorporated herein by reference in their entirety.

20 WO2012/154530 describes that the addition of a short, flexible linker of between 3 and 8 amino acids each independently selected from glycine and serine between the FCS and the PE functional domain III improves the cytotoxicity of the PE-LR molecule without disrupting binding by furin. Exemplary linkers are GGS and GGSGGS (SEQ ID NO: 7).

Other work has sought to further reduce the immunogenicity of PE.

30 WO2012/154530 reports that substitutions at the following amino acid residues within PE domain III reduce immunogenicity:

D403, D406, R412, R427, E431, R432, R458, D461, R467, R490, R505, R513, E522, R538, E548, R551, R576, K590, Q592 and L597.

35 Preferred substitutions are with a glycine, serine or alanine residue.

WO2012/170617 reports that substitutions at these residues may reduce immunogenicity of B cell epitopes, and that substitutions at one or more of residues R427, R458, R467, R490, R505 and F538 are preferred, particularly 40 with alanine.

WO2013/040141 reports that substitutions at the following additional amino acid residues may reduce the immunogenicity of B cell epitopes within PE domain III:

5 E420, D463, Y481, L516, R563, D581, D589 and K606.

Preferred substitutions are with a glycine, serine, alanine or glutamine residue.

10 WO2012/170617 reports that substitutions at the following residues can reduce the immunogenicity of T-cell epitopes within PE domain III:

R421, L422, L423, A425, R427, L429, Y439, H440, F443, L444, A446,
A447, I450, 463-519, R551, L552, T554, I555, L556 and W558.

15 Preferred substitutions are at one or more of residues D463, Y481 and L516, which may also reduce the immunogenicity of B cell epitopes. Preferred substitutions are with a glycine, serine, alanine or glutamine residue.

20 WO2012/170617 also reports that substitutions at the following amino acid residues can reduce the immunogenicity of T cell epitopes within PE domain II:

L294, L297, Y298, L299 and R302.

25 Preferred substitutions are with a glycine, serine, alanine or glutamine residue.

30 WO2012/170617 also reports that substitutions at the following amino acid residues can reduce the immunogenicity of B cell epitopes within PE domain II:

E282, E285, P290, R313, N314, P319, D324, E327, E331 and Q332.

35 WO2012/170617 also reports that a particularly preferred combination of substitutions is D463A/R427A/R458A/R467A/R490A/R505A/R538A.

Alewine et al. discloses a similar combination of 7 point mutations within PE domain III that reduce B-cell immunogenicity, namely

40 R427A/R456A/D463A/R467A/R490A/R505A/R538A (that is, with R456A instead of R458A).

Mazor et al. discloses that a combination of 6 point mutations within PE domain III, together with deletion of most of PE domain II, reduced T cell responses by 93%. The mutations are R427A/F443A/L477H/R494A/R505A/L552E.

5

Accordingly, the PE functional domain III may comprise mutations at any one or any combination of more than one of the following sites:

10

D403, D406, R412, E420, R421, L422, L423, A425, R427, L429, E431, R432, Y439, H440, F443, L444, A446, A447, I450, R456, R458, D461, 463-519 (preferably D463, R467, L477, Y481, R490, R494, R505, R513 and/or L516), E522, R538, E548, R551, L552, T554, I555, L556, W558, R563, R576, D581, D589, K590, Q592, L597 and K606.

15

Preferably the mutation(s) reduce(s) the immunogenicity compared to the unmutated sequence of the amino acids 395-613 of SEQ ID NO:1.

Insofar as the PE contains some or all of domain II, it may comprise mutations at any one or any combination of more than one of the following sites:

20

E282, E285, P290, L294, L297, Y298, L299, R302, R313, N314, P319, D324, E327, E331 and Q332.

25

Preferably the mutation(s) reduce(s) the immunogenicity compared to the unmutated sequence from domain II.

In particular, in embodiments in which the FCS is derived from the native furin-cleavable sequence of PE consisting of amino acids 274-284

30

(RHRQPRGWEQL, SEQ ID NO: 6) may comprise a substitution of the E282 residue, especially if the adjacent sequence from the native PE sequence is included downstream of the FCS. In embodiments where the adjacent sequence from the native PE sequence is not included (such as PE-LR, in which the FCS is fused to domain III either directly or via a non-native linker sequence), the epitope from the native sequence may anyway be disrupted such that a mutation at the E282 residue may not be advantageous.

35

Mazor et al. 2014, Liu et al. 2012, Kreitman et al. 2012, Pastan et al. 2011, Onda et al. 2011, Hansen et al. 2010, Kreitman et al. 2009c, Onda et al. 2008, Ho et al. 2005, Kreitman et al. 2000 and Roscoe et al. 1994 are all directed towards reducing the immunogenicity of PE.

40

Reduced immunogenicity in variant PE toxins may refer to a reduced ability of the variant sequence to induce a T cell response and/or a reduced ability of the variant sequence to induce a B cell (antibody) response, preferably both. Techniques for assessing the effect of mutations on T cell immunogenicity are well known in the art and described in the examples of WO 2012/170617. Techniques for assessing the effect of mutations on the B cell immunogenicity are likewise well known in the art and described in WO 2013/040141, for example. Human antibodies may be raised against the native PE sequence by phage display using a human antibody library. The ability of mutations in the PE sequence to disrupt binding of such antibodies to the variant PE molecule is indicative of reduced immunogenicity. Alternatively, the titre of PE-specific antibodies raised in transgenic mice carrying the human antibody repertoire may be compared for the native and mutated PE sequences.

The C-terminal end of the PE functional domain III may contain the native sequence of residues 609-613, namely REDLK (SEQ ID NO: 8). Additionally or alternatively to any other modifications of the native PE sequence, the PE functional domain III may contain a variant of the REDLK (SEQ ID NO: 8) sequence, or other sequences, that function to maintain the PR protein in the endoplasmic reticulum or to recycle proteins into the endoplasmic reticulum. Such sequences are referred to here as "endoplasmic reticulum localisation sequences" or "ER localisation sequences". Preferred ER localisation sequences include such as KDEL (SEQ ID NO: 9), REDL (SEQ ID NO:10), RDEL (SEQ ID NO:11) or KEDLK (SEQ ID NO: 12). One or more additional ER localisation sequences, preferably independently selected from KDEL (SEQ ID NO: 9), REDL (SEQ ID NO: 10), REDLK (SEQ ID NO: 8), RDEL (SEQ ID NO:11) and KEDLK (SEQ ID NO:12), may be added to the C-terminal end of the PE polypeptide sequence. The substitution of KDEL (SEQ ID NO: 9), or 2 or 3 tandem repeats of KDEL (SEQ ID NO: 9) (KDELKDEL, SEQ ID NO 13; KDELKDELKDEL, SEQ ID NO 14) for the native REDLK (SEQ ID NO: 8) sequence, or the addition of KDEL (SEQ ID NO: 9) after the native REDLK (SEQ ID NO: 8) sequence is preferred. See for example WO91/099949, Chaudhary et al 1991 Seetharam et al 1991.

WO91/09949 discloses that the C-terminal end of the PE functional domain III may lack some or all of residues 602-608, which are not essential for the NAD(+)-diphthamide ADP ribosyltransferase activity.

Furin-cleavable sequence (FCS)

As described in WO2012/154530, the furin-cleavable sequence can be any polypeptide sequence cleavable by furin. Duckert et al. 2004, Protein Engineering, Design & Selection 17(1):107-112 (hereafter, "Duckert et al.")

5 is incorporated herein by reference in its entirety and particularly with regard to the furin-cleavable sequences and motifs it discloses. Duckert et al. discloses that furin is an enzyme in a family of evolutionarily conserved dibasic- and monobasic-specific CA²⁺-dependent serine proteases called subtilisin/kexin-like proprotein convertases. See page 107.

10 Furin, also known as "paired basic amino acid cleaving enzyme", "PACE", or PCSK3, is one of several mammalian members of the PCSK family and is involved in processing several endogenous human proteins. See generally, Thomas 2002 Nat Rev Mol Cell Biol 10:753-66. It is a membrane-associated protein found mainly in the trans-Golgi network. The sequence of human
15 furin has been known since the early 1990s. See for example Hatsuzawa et al. 1992 J Biol Chem 267: 16094-16099; and Molloy et al. 1992 J Biol Chem 267:16396-16402.

The minimal furin-cleavable sequence typically is, in the single letter
20 code for amino acid residues, R-X-X-R (SEQ ID NO:15), with cleavage occurring after the second "R". Duckert et al. summarizes the information available on the sequences of 38 proteins reported in the literature to have furin-cleavable sequences, including mammalian proteins, proteins of pathogenic bacteria, and viral proteins. It reports that 31, or 81%, of the
25 cleavage motifs reviewed had the R-X-[R/K]-R (SEQ ID NOs:16 & 17) consensus sequence, of which 11, or 29%, had R-X-R-R (SEQ ID NO:16), and 20, or 52%, were R-X-K-R (SEQ ID NO:17). Three of the cleavage motifs contained only the minimal cleavage sequence. Duckert et al. further aligned the motifs and identified the residues found at each position in each furin both for
30 the cleavage motif itself and in the surrounding residues. Fig. 1A of Duckert et al. shows by relative size the residues most commonly found at each position. By convention, the residues surrounding the furin cleavage site are numbered from the scissile bond (which is typically indicated by a downward arrow). Counting toward the N terminus, the substrate residues
35 are designated P1, P2, and so on, while counting towards the C-terminus, the residues are designated P1', P2', and so on. See Rockwell and Thorner 2004 Trends Biochem. Sci. 29:80-87; and Thomas 2002 Nat. Rev. Mol. Cell Biol 3:753-766. Thus, following the convention, the following sequence can be used to align and number the residues of the minimal cleavage sequence
40 and the surrounding residues:

P6-P5-P4-P3-P2-P1-P1'-P2'-P3'-P4'-P5',

in which the minimal furin-cleavable sequence is numbered as P4-P1. Duckert et al.'s alignment of 38 sequences cleaved by furin identifies the variations permitted depending on the residues present at various positions. For example, if the residue at P4 is not an R, that can be compensated for by having arginine or lysine residues at P2 and P6. See page 109.

In native PE, furin cleavage occurs between arginine 279 and glycine 280 in an arginine-rich loop located in domain II of the toxin. The native furin-cleavable sequence in domain II of PE is set forth below (with numbers indicating the positions of the residues in the 613-amino acid native PE sequence), and aligned to show its numbering under the convention noted above:

274- R H R Q P R G W E Q L -284 (SEQ ID NO:6)
 P6--P5--P4--P3--P2--P1--P1'-P2'-P3'-P4'-P5'

In studies underlying WO2012/154530, substitutions were made at positions P3 and P2 to form the following sequence, with the substitutions underlined:

274- R H R S K R G W E Q L -284 (SEQ ID NO: 18).

This sequence has shown a cleavage rate faster than that of the native sequence, and when used in an exemplary immunotoxin resulted in cytotoxicity to target cells approximately the same as that of the native sequence.

Based on this and previous studies, a furin-cleavable sequence used to attach the targeting molecule to PE domain III can be the minimal furin-cleavable sequence, R-X-X-R (SEQ ID NO: 15) (wherein each X is independently any naturally occurring amino acid), preferably R-X-[R/K]-R (SEQ ID Nos: 16 & 17) (wherein X is any naturally occurring amino acid and [R/K] denotes either arginine or lysine), or any of the other furin-cleavable sequences known in the art or permitted by Fig. 1 A of Duckert et al., with the proviso that, if there is a residue present at the position identified as P2', it should be tryptophan or, if not tryptophan, should not be valine or alanine. For example, in some embodiments, the sequence can be RKKR (SEQ ID NO:19), RRRR (SEQ ID NO:20), RKAR (SEQ ID NO: 21),

SRVARS (SEQ ID NO: 22), TSSRKRRFW (SEQ ID NO: 23), or ASRRKARSW (SEQ ID NO: 24).

As noted in Duckert et al., a less favorable residue than R (primarily valine) can be used position P4 if compensated for by arginine or lysine residues at positions P2 and P6, so that at least two of the three residues at P2, P4 and P6 are basic. Thus, in some embodiments, the furin-cleavable sequence is RRVKKRFW (SEQ ID NO: 25), RNVVRRDW (SEQ ID NO: 26), or TRAVRRRSW (SEQ ID NO: 27). The residue at position PI can be the arginine present in the native sequence, or lysine. Thus, a lysine can be substituted for the arginine at position PI in, for example, any the sequences set forth above.

In some embodiments, the furin-cleavable sequence contains the native furin-cleavable sequence of PE: R-H-R-Q-P-R-G-W-E-Q-L (SEQ ID NO: 6) or a truncated version of the native sequence, so long as it contains the minimal furin-cleavable sequence and is cleavable by furin. Thus, in some embodiments, the furin-cleavable sequence can be R-Q-P-R (SEQ ID NO: 28), R-H-R-Q-P-R-G-W (SEQ ID NO: 29), R-H-R-Q-P-R-G-W-E (SEQ ID NO: 30), H-R-Q-P-R-G-W-E-Q (SEQ ID NO: 31), or R-Q-P-R-G-W-E (SEQ ID NO: 32). In some embodiments, the sequence is R-H-R-S-K-R-G-W-E-Q-L (SEQ ID NO: 18) or a truncated version of this sequence, so long as it contains the minimal furin-cleavable sequence and is cleavable by furin. Thus, in some embodiments, the furin-cleavable sequence can be R-S-K-R (SEQ ID NO: 33), R-H-R-S-K-R-G-W (SEQ ID NO: 34), H-R-S-K-R-G-W-E (SEQ ID NO: 35), R-S-K-R-G-W-E-Q-L (SEQ ID NO: 36), H-R-S-K-R-G-W-E-Q-L (SEQ ID NO: 37), or R-H-R-S-K-R (SEQ ID NO: 38).

As mentioned above, the E282 residue at the P3' position of FCS sequences derived from PE may be replaced by another amino acid to reduce B cell immunogenicity. Where the sequence lacks native PE residues downstream of this residue, or where the FCS contains other mutations relative to the native PE sequence, such replacement may not be necessary.

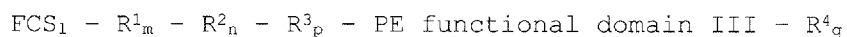
Whether or not any particular sequence is cleavable by furin can be determined by methods known in the art. For example, whether or not a sequence is cleavable by furin can be tested by incubating the sequence with furin in furin buffer (0.2 M NaOAc (pH 5.5), 5 mM CaCl₂) at a 1 :10 enzyme:substrate molar ratio at 25°C for 16 hours. These conditions have previously been established as optimal for furin cleavage of PE. Preferably, the furin used is human furin. Recombinant truncated human

furin is commercially available, for example, from New England Biolabs (Beverly, MA). See also, Bravo et al. 1994 J Biol Chem 269(14):25830-25837.

Alternatively, a furin-cleavable sequence can be tested by making it into an immunotoxin with an antibody against a cell surface protein and testing the resulting immunotoxin on a cell line expressing that cell surface protein. Suitable antibody sequences are disclosed in, for example, WO2012/154530 and WO2009/032954.

General formula for preferred PE toxins

Preferred PE toxins for use in accordance with the present invention have the following structure:



wherein:

l, m, n, p and q are each, independently, 0 or 1;

FCS is a furin-cleavable sequence, preferably (i) R-H-R-Q-P-R-G-W-E-Q-L (SEQ ID NO: 6) or a truncated version thereof containing R-Q-P-R (SEQ ID NO: 28), optionally R-Q-P-R (SEQ ID NO: 28), R-H-R-Q-P-R-G-W (SEQ ID NO: 29), R-H-R-Q-P-R-G-W-E (SEQ ID NO: 30), H-R-Q-P-R-G-W-E-Q (SEQ ID NO: 31), or R-Q-P-R-G-W-E (SEQ ID NO: 32); or (ii) R-H-R-S-K-R-G-W-E-Q-L (SEQ ID NO: 18) or a truncated version thereof containing R-S-K-R (SEQ ID NO: 33), optionally R-S-K-R (SEQ ID NO: 33), R-H-R-S-K-R-G-W (SEQ ID NO: 34), H-R-S-K-R-G-W-E (SEQ ID NO: 35), R-S-K-R-G-W-E-Q-L (SEQ ID NO: 36), H-R-S-K-R-G-W-E-Q-L (SEQ ID NO: 37), or R-H-R-S-K-R (SEQ ID NO: 38), wherein the glutamic acid residue corresponding to position 282 of the native PE sequence (where present) is optionally replaced by another residue, preferably glycine, serine, alanine or glutamine;

R^1 is a linker sequence of 1 to 10 amino acids, preferably GGS or GGSGGS (SEQ ID NO: 7);

R^2 is one or more consecutive amino acid residues of residues 285-364 of SEQ ID NO:1, in which any one or more of residues E285, P290, L294, L297, Y298, L299, R302, R313, N314, P319, D324, E327, E331 and Q332, where present, is/are optionally independently replaced by another amino acid, preferably glycine, serine, alanine or glutamine;

R^3 is one or more consecutive amino acid residues of residues 365-394 of SEQ ID NO:1;

PE functional domain III comprises residues 395-613 of SEQ ID NO:1 in which:

(a) some or all of residues 602-608 are optionally deleted, and

(b) residues 609-613 are optionally replaced by another ER localisation sequence, preferably KDEL (SEQ ID NO: 9), REDL (SEQ ID NO:10), RDEL (SEQ ID NO:11) or KEDLK (SEQ ID NO:12), and

(c) any one or more of residues D403, D406, R412, E420, R421, L422, L423, A425, R427, L429, E431, R432, Y439, H440, F443, L444, A446, A447, I450, R456, R458, D461, 463-519 (preferably D463, R467, L477, Y481, R490, R494, R505, R513 and/or L516), E522, R538, E548, R551, L552, T554, I555, L556, W558, R563, R576, D581, D589, K590, Q592, L597 and (where present) K606 is/are optionally independently replaced by another amino acid, preferably glycine, serine, alanine or glutamine, or histidine in the case of L477;

R⁴ is one or more (preferably 1 or 2) additional ER localisation sequences, preferably REDLK (SEQ ID NO: 8), KDEL (SEQ ID NO: 9), REDL (SEQ ID NO:10), RDEL (SEQ ID NO:11) or KEDLK (SEQ ID NO:12).

Within the formula above:

l is preferably 1; that is, an FCS is preferably present;

m is preferably 1; that is, a linker is preferably present especially in the case that l is 1;

n is preferably 0; that is, residues 285-364 of SEQ ID NO:1 are preferably absent;

p is preferably 0; that is residues 365-394 of SEQ ID NO:1 are preferably absent;

PE functional domain III preferably includes the combination of mutations R427A/F443A/L477H/R494A/R505A/L552E, or the combination of mutations R427A/R456A/D463A/R467A/R490A/R505A/R538A, or the combination of mutations

R427A/F443A/R456A/D463A/R467A/L477H/R490A/R494A/R505A/R538A/L552E.

Particularly preferred PE toxins for use in accordance with the present invention comprise the amino acid sequence of SEQ ID NO:64 or SEQ ID NO:65. SEQ ID NO:64 corresponds to amino acid residues 395-613 of SEQ ID NO:1 with Ala substitutions at positions 427, 456, 463, 467, 490, 505 and 538 and is disclosed in WO2015/51199 as LO10R-456A and SEQ ID NO:37. SEQ ID NO:65 corresponds to amino acid residues 395-613 of SEQ ID NO:1 with Ala substitutions at positions 427, 443, 477, 494, 505 and 552 and is disclosed in WO2015/051199 as T18/T20 and SEQ ID NO:289.

The amino acid sequences of SEQ ID NO:64 and SEQ ID NO:65 are each preferably fused to the C-terminal end of the amino acid sequence of SEQ ID NO:66, which corresponds to SEQ ID NO:36 of WO2015/051199 and contains an FCS and linker sequences.

5

Diphtheria toxins (DTs)

Native, wild-type *Diphtheria* toxin is secreted by *Corynebacterium diphtheriae* and has the 535 amino acid sequence shown in SEQ ID NO:3. This sequence is shown without the native signal peptide, which is shown as the first 25 amino acids of UniProt accession number Q6NK15, version 1.

10

The order of the structural domains in DT is reversed compared to that of PE. That is, the N-terminal domain I (amino acids 1-191 of SEQ ID NO:3) is NAD(+)-diphthamide ADP ribosyltransferase domain; and the C-terminal domain III (amino acids 385-535 of SEQ ID NO:3) is the cell-binding domain. As with PE, activation of the native DT protein depends on furin cleavage within domain II.

15

Truncated or modified forms of DT that lack receptor-binding activity (truncated: DAB₃₈₉, DAB₄₈₆, DT₃₈₅, DT₃₈₈, DT₃₉₀; modified: CRM107; point mutation of S525F) have been widely used in the form of immunotoxins, coupled to other targeted therapeutic agents. Exemplary truncated forms of DT include residues 1-384, 1-387, 1-388, 1-389 or 1-485 of SEQ ID NO:3), optionally with an additional N-terminal methionine residue from recombinant expression in bacterial cells.

20

25

Generally, a DT toxin will have a polypeptide sequence comprising a DT functional domain I having at least 50% amino acid sequence identity over the full length of residues 1-191 of SEQ ID NO:3 and having cytotoxic activity when introduced into a eukaryotic (preferably mammalian) cell. Preferred forms of DT comprise a DT functional domain I having at least 50% amino acid sequence identity over the full length of residues 1-191 of SEQ ID NO:3 and having NAD(+)-diphthamide ADP ribosyltransferase activity.

30

In embodiments in which the DT is coupled to a cell-binding agent as a fusion polypeptide, the DT preferably also comprises (2) a cleavable linker sequence such as a furin-cleavable sequence (FCS) that permits cleavage of the DT functional domain I from the cell-binding agent following uptake into the target cell.

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The cleavable linker (such as an FCS) will generally be on the C-terminal side of the DT functional domain I. The furin-cleavable sequence preferably includes the minimal furin-cleavable sequence motif from the native DT sequence, namely the R-V-R-R (SEQ ID NO: 39) sequence at residues 190-193 of SEQ ID NO:3. It may also include N-terminal and/or C-terminal flanking regions from the native sequence. Preferably the furin-cleavable sequence includes the sequence GNRVRRSVGSS (SEQ ID NO: 40) or a fragment thereof comprising RVRR (SEQ ID NO: 39). As with PE toxins, however, other cleavable linkers and other means of coupling the DT to cell-binding agents are contemplated.

In contrast to PE, it is thought that DT is not processed in target cells via the endoplasmic reticulum, so a DT for use in accordance with the invention will generally lack an ER localisation sequence.

As with PE, DT may contain deletions within domain II, particularly downstream of the furin-cleavable sequence (that is, within residues 194-384 of SEQ ID NO:3, preferably within residues 200-384 so as to preserve a longer native furin-cleavable sequence). As with PE, the DTs for use in accordance with the invention may also be mutated to reduce immunogenicity.

The DT for use in accordance with the present invention will generally lack a functional cell-binding domain III.

Cholix toxins

Native, wild-type cholix toxin is secreted by *Vibrio cholerae* and has the 634 amino acid sequence shown in SEQ ID NO:4. This sequence is shown without the native signal peptide, which is shown as the first 32 amino acids of UniProt accession number Q5EK40.1 (gi:75355041).

The order of the structural domains in cholix toxin is the same as that of PE. That is, domain Ia (amino acids 1-264) is the cell-binding domain; domain II (amino acids 265-386) is the translocation domain and contains the furin-cleavable sequence RKPR (SEQ ID NO: 41) at residues 289-292; domain Ib (amino acids 387-423) is of unknown function and domain III (amino acids 424-634) is the catalytic domain, comprising an ER localisation sequence RKDELK (SEQ ID NO: 42) at positions 629-634. See Awasthi et al. 2013, which also provides naturally occurring variant cholix toxin sequences.

Generally, a cholix toxin will have a polypeptide sequence comprising a cholix toxin functional domain III having at least 50% amino acid sequence identity over the full length of residues 424-628 of SEQ ID NO:4, wherein the cholix toxin has cytotoxic activity when introduced into a eukaryotic (preferably mammalian) cell. Preferred forms of cholix toxin comprise (1) a cholix toxin functional domain III having at least 50% amino acid sequence identity over the full length of residues 424-628 of SEQ ID NO:4 and having NAD(+)-diphthamide ADP ribosyltransferase activity, and (2) at least one endoplasmic reticulum localisation sequence.

In embodiments in which the cholix toxin is coupled to a cell-binding agent as a fusion polypeptide, the cholix toxin preferably also comprises (3) a cleavable linker sequence such as a furin-cleavable sequence (FCS) that permits cleavage of the cholix toxin functional domain III from the cell-binding agent following uptake into the target cell. The cleavable linker (such as an FCS) will generally be on the N-terminal side of the cholix toxin functional domain III.

The furin-cleavable sequence preferably includes the minimal furin-cleavable sequence motif from the native cholix toxin sequence, namely the RKPR (SEQ ID NO: 41) sequence of residues 289-292 of SEQ ID NO:4. It may also include N-terminal and/or C-terminal flanking regions from the native sequence. Preferably the furin-cleavable sequence includes the sequence RSRKPRDLTDD (SEQ ID NO: 43) of amino acids 287-297 of SEQ ID NO:4 or a fragment thereof comprising RKPR (SEQ ID NO: 41). Alternatively, it may comprises the sequence RGRKPRDLTDD (SEQ ID NO: 44) of ChxA III of Awasthi et al. 2013 or a fragment thereof comprising RKPR (SEQ ID NO: 41). Alternatively, it may comprises the sequence RSRKPRDLPDD (SEQ ID NO:45) of ChxA I and II of Awasthi et al. 2013 or a fragment thereof containing RKPR (SEQ ID NO: 41). As with PE toxins, however, other cleavable linkers and other means of coupling the cholix toxin to cell-binding agents are contemplated.

The ER localisation sequence may be the native RKDELK (SEQ ID NO: 42) sequence of SEQ ID NO:4 or the HDELK (SEQ ID NO: 46) sequence of ChxA III of Awasthi et al. 2013 or any of the ER localisation sequences disclosed above for PE. As with PE, the cholix toxin may include one or more additional ER localisation sequences.

As with PE, the cholix toxin may include some or all of domain Ib (amino acids 387-423), preferably at least about 10 amino acids from the C-terminus of domain Ib, that is, at least about amino acids 413-422.

- 5 The cholix toxins for use in accordance with the present invention will generally lack a functional cell-binding domain I.

- As with PE, cholix toxin may contain deletions, particularly upstream of the furin-cleavable sequence and/or between the furin-cleavable sequence and the cholix toxin domain III (that is, within residues 1-288 (preferably 1-286) and/or 293-423 (preferably 298-423 or 293-413, more preferably 298-413) of SEQ ID NO:4.
- 10

- As with PE, the cholix toxins for use in accordance with the invention may also be mutated to reduce immunogenicity.
- 15

Cytotoxic activity

- Confirmation or comparison of the cytotoxic activity of the NAD(+)-diphthamide ADP-ribosyltransferases of the present invention may be tested using a cytotoxic activity assay. The NAD(+)-diphthamide ADP-ribosyltransferase is coupled to a cell-binding agent that is targeted to the cells used in the assay. A wide variety of cytotoxicity assays are available, such as the WST assay used in WO 2011/032022, which measures cell proliferation using the tetrazolium salt WST-1. Reagents and kits are commercially available from Roche Applied Sciences.
- 20
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- 30 *NAD(+)-diphthamide ADP ribosyltransferase activity*
- NAD(+)-diphthamide ADP-ribosyltransferase activity may be assayed by the ability to incorporate biotinylated ADP into eEF2 protein, as described in Example 4 herein.

- 35 *Exemplary targeted therapeutic agents*

Immunotoxins that combine an antibody with a PE toxin and that have progressed to clinical trials are reviewed in Weldon & Pastan (2011) and include the following:

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1. RFB4(dsFv)PE38 (also known as BL22 or CAT-3888) directed against CD22, for the treatment of B-cell malignancies (Kreitman et al. 2005, Kreitman et al. 2009a, Wayne et al. 2010).
2. A second generation, affinity matured version of (1), moxetumomab pasudotox (also known as RFB4[GTHW](dsFv)-PE38, HA22 or CAT-8015) (Salvatore et al. 2002), for the treatment of hematologic malignancies (Alderson et al. 2009, Kreitman et al. 2009b, ClinicalTrial.gov identifiers NCT00462189, NCT00457860, NCT00515892, NCT01086644, NCT00659425, and NCT00586924).
3. SS1(dsFv)PE38 (also known as SS1P) directed against mesothelin, for the treatment of lung cancer and mesothelioma (Hassan et al., Kreitman et al. 2009c, ClinicalTrial.gov identifiers NCT01041118, NCT00575770, and NCT01051934)
4. anti-TAC(scFv)PE38 (also known as LMB-2) directed against IL-2R, for the treatment of hematologic malignancies (Kreitman et al. 2000, ClinicalTrial.gov identifiers NCT00924170, NCT00077922, NCT00080535, and NCT00321555).

Wolf et al. 2009 and Shapira et al. 2010 review other targeted therapeutic agents in pre-clinical and clinical development, which combine either an antibody or another cell-binding agent with a PE or DT toxin. In particular, Table 1 of Shapira et al. 2010 refers to targeted therapeutic agents incorporating a variety of truncated or modified forms of DT that lack receptor-binding activity (truncated: DAB₃₈₉, DAB₄₈₆, DT₃₈₈, DT₃₉₀; modified: CRM107) or a variety of PE toxins (full-length PE, PE38, PE40, modified PE38 and modified PE40), coupled to a variety of cell-binding agents (IL-2, transferrin, GM-CSF, EGF, anti-CD3 ϵ , variant IL-3, anti-ovarian antigen, anti-HER2, anti-mesothelin, anti-Lewis Y, anti-CD22, anti-CD25, TGF α , circularly permuted IL-4 and IL-13) for a variety of indications (leukaemia and lymphoma, including cutaneous T cell lymphoma (CTCL), non-Hodgkin's lymphoma (NHL), chronic lymphoblastic lymphoma (CLL), Hodgkin's disease (HD), small lymphatic lymphoma (SLL), prolymphocytic leukaemia (PLL), acute myelogenous leukaemia (AML), hairy cell leukaemia (HCL), acute lymphoblastic leukaemia (ALL) and T-cell lymphoma/leukaemia; lung cancer including non-small cell lung cancer (NSCLC) and mesothelioma; other cancers including adenocarcinoma, EGFR-expressing carcinomas, melanoma, ovarian cancer, breast cancer, kidney cancer, Kaposi's sarcoma (KS), brain and CNS tumours, oesophageal cancer, pancreatic cancer, colon cancer, bladder cancer, glioblastoma, and glioma; graft-versus host disease (GVHD); psoriasis; Rheumatoid arthritis (RA); and myelodysplastic syndrome (MDS). Any of these targeted therapeutic agents may be used in accordance

with the present invention. Furthermore, the cell-binding agents of these targeted therapeutic agents may be used with other NAD(+)-diphthamide ADP ribosyltransferases, especially for the indications shown. Still further cell-binding agents are disclosed in the context of other (non-NAD(+)-
5 diphthamide ADP ribosyltransferase) toxins and may similarly be used with NAD(+)-diphthamide ADP ribosyltransferases, especially for the indications shown. Shapira et al. is incorporated herein by reference in its entirety and (along with the references cited in Table 1) especially for the purpose of exemplifying both specific targeted therapeutic agents and cell-binding
10 agents and their associated indications, suitable for use in accordance with the present invention.

Targeted therapeutic agents

15 For therapeutic use, the NAD(+)-diphthamide ADP ribosyltransferases herein are coupled to a cell binding agent to produce a targeted therapeutic agent. The term "targeted therapeutic agent" is used in the broadest sense and is not intended to imply that the cell binding agent is necessarily an antibody or immunoglobulin. As discussed below, a wide variety of cell
20 binding agents may be included in targeted therapeutic agents in accordance with the invention.

Where the cell binding agent is a peptide, polypeptide, or protein, the NAD(+)-diphthamide ADP ribosyltransferase is preferably coupled to the cell
25 binding agent as a fusion polypeptide or protein. Fusion may be direct or via a linker peptide. The fusion polypeptide or protein may be produced recombinantly, avoiding any need for conjugation chemistry.

When the NAD(+)-diphthamide ADP ribosyltransferase fused to the cell binding
30 agent comprises a furin-cleavable sequence, the furin-cleavable sequence will generally be positioned between the cell binding agent and the cytotoxic domain of the NAD(+)-diphthamide ADP ribosyltransferase, such that cleavage of the fusion polypeptide inside the target cell will separate the cytotoxin domain from the cell binding agent. In preferred embodiments of
35 the invention, the NAD(+)-diphthamide ADP ribosyltransferase will be positioned on the C-terminal side of the cell binding agent.

Alternatively, however, the NAD(+)-diphthamide ADP ribosyltransferase may
40 be conjugated to the cell binding agent.

Cell binding agents

A cell binding agent may be of any kind, and include peptides and non-peptides. These can include antibodies or a fragment of an antibody that contains at least one binding site, lymphokines, hormones, growth factors,
5 nutrient-transport molecules, or any other cell binding molecule or substance.

The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, dimers, multimers,
10 multispecific antibodies (e.g., bispecific antibodies), and antibody fragments, so long as they exhibit the desired biological activity (Miller et al (2003) *Jour. of Immunology* 170:4854-4861). Antibodies may be murine, human, humanized, chimeric, or derived from other species. An antibody is a protein generated by the immune system that is capable of recognizing and
15 binding to a specific antigen. (Janeway, C., Travers, P., Walport, M., Shlomchik (2001) *Immuno Biology, 5th Ed.*, Garland Publishing, New York). A target antigen generally has numerous binding sites, also called epitopes, recognized by CDRs on multiple antibodies. Each antibody that specifically binds to a different epitope has a different structure. Thus, one antigen
20 may have more than one corresponding antibody. An antibody includes a full-length immunoglobulin molecule or an immunologically active portion of a full-length immunoglobulin molecule, i.e., a molecule that contains an antigen binding site that immunospecifically binds an antigen of a target of interest or part thereof, such targets including but not limited to,
25 cancer cell or cells that produce autoimmune antibodies associated with an autoimmune disease. The immunoglobulin can be of any type (e.g. IgG, IgE, IgM, IgD, and IgA), class (e.g. IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. The immunoglobulins can be derived from any species, including human, murine, or rabbit origin.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies; fragments produced by a Fab expression library, anti-
35 idiotypic (anti-Id) antibodies, CDR (complementary determining region), and epitope-binding fragments of any of the above which immunospecifically bind to cancer cell antigens, viral antigens or microbial antigens, single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Particularly preferred antibody fragments for use in accordance
40 with the invention include include Fab fragments, scFv fragments and disulphide-stabilised Fv fragments, especially Fab fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e. the individual antibodies comprising the population are identical except
5 for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations which include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a
10 single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as
15 requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al (1975) *Nature* 256:495, or may be made by recombinant DNA methods (see, US 4816567). The monoclonal antibodies may also be isolated
20 from phage antibody libraries using the techniques described in Clackson et al (1991) *Nature*, 352:624-628; Marks et al (1991) *J. Mol. Biol.*, 222:581-597.

The monoclonal antibodies herein specifically include "chimeric" antibodies
25 in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or
30 belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (US 4816567; and Morrison et al (1984) *Proc. Natl. Acad. Sci. USA*, 81:6851-6855). Chimeric antibodies include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate
35 (e.g. Old World Monkey or Ape) and human constant region sequences.

An "intact antibody" herein is one comprising VL and VH domains, as well as a light chain constant domain (CL) and heavy chain constant domains, CH1, CH2 and CH3. The constant domains may be native sequence constant domains
40 (e.g. human native sequence constant domains) or amino acid sequence variant thereof. The intact antibody may have one or more "effector

functions" which refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody. Examples of antibody effector functions include Clq binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; and down regulation of cell surface receptors such as B cell receptor and BCR.

Depending on the amino acid sequence of the constant domain of their heavy chains, intact antibodies can be assigned to different "classes." There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into "subclasses" (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called α , δ , ϵ , γ , and μ , respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The cell binding agent may be, or comprise, a polypeptide. The cell binding agent is preferably an antibody. Where the cell-binding agent is a peptide, the peptide may comprise 4-20, preferably 6-20, contiguous amino acid residues.

Antibodies for use in the targeted therapeutic agents of the invention include those antibodies described in WO 2005/082023 which is incorporated herein. Particularly preferred are those antibodies for tumour-associated antigens. Examples of those antigens known in the art include, but are not limited to, those tumour-associated antigens set out in WO 2005/082023. See, for instance, pages 41-55.

The cell-binding agents described herein are designed to target diseased cells such as tumour cells via their cell surface antigens. The antigens are usually normal cell surface antigens which are either over-expressed or expressed at abnormal times. Ideally the target antigen is expressed only on diseased cells (such as tumour cells), however this is rarely observed in practice. As a result, target antigens are usually selected on the basis of differential expression between diseased and healthy tissue.

Thus, the cell-binding agent may specifically bind to any suitable cell surface marker. The choice of a particular targeting moiety (cell-binding agent) and/or cell surface marker may be chosen depending on the particular cell population to be targeted. Cell surface markers are known in the art

(see, e.g., Mufson et al., *Front. Biosci.*, 11:337-43 (2006); Frankel et al., *Clin. Cancer Res.*, 6:326-334 (2000); and Kreitman et al., *AAPS Journal*, 8(3): E532-E551 (2006)) and may be, for example, a protein or a carbohydrate. In an embodiment of the invention, the targeting moiety
5 (cell-binding agent) is a ligand that specifically binds to a receptor on a cell surface. Exemplary ligands include, but are not limited to, vascular endothelial growth factor (VEGF), Fas, TNF-related apoptosis-inducing ligand (TRAIL), a cytokine (e.g., IL-2, IL-15, IL-4, IL-13), a lymphokine, a hormone, and a growth factor (e.g., transforming growth factor (TGF α),
10 neuronal growth factor, epidermal growth factor).

The cell surface marker can be, for example, a tumour-associated antigen. The term "tumour-associated antigen" as used herein refers to any molecule (e.g., protein, peptide, lipid, carbohydrate, etc.) solely or predominantly
15 expressed or over-expressed by tumour cells and/or cancer cells, such that the antigen is associated with the tumour(s) and/or cancer(s). The tumour-associated antigen can additionally be expressed by normal, non-tumour, or non-cancerous cells. However, in such cases, the expression of the tumour-associated antigen by normal, non-tumour, or non-cancerous cells is not as
20 robust as the expression by tumour or cancer cells. In this regard, the tumour or cancer cells can over-express the antigen or express the antigen at a significantly higher level, as compared to the expression of the antigen by normal, non-tumour, or non-cancerous cells. Also, the tumour-associated antigen can additionally be expressed by cells of a different
25 state of development or maturation. For instance, the tumour-associated antigen can be additionally expressed by cells of the embryonic or fetal stage, which cells are not normally found in an adult host. Alternatively, the tumour-associated antigen can be additionally expressed by stem cells or precursor cells, which cells are not normally found in an adult host.

The tumour-associated antigen can be an antigen expressed by any cell of any cancer or tumour, including the cancers and tumours described herein. The tumour-associated antigen may be a tumour-associated antigen of only
30 one type of cancer or tumour, such that the tumour-associated antigen is associated with or characteristic of only one type of cancer or tumour. Alternatively, the tumour-associated antigen may be a tumour-associated antigen (e.g., may be characteristic) of more than one type of cancer or tumour. For example, the tumour-associated antigen may be expressed by both
35 breast and prostate cancer cells and not expressed at all by normal, non-tumour, or non-cancer cells.
40

Exemplary tumour-associated antigens to which the cell-binding agent may specifically bind include, but are not limited to, mucin 1 (MUC1; tumour-associated epithelial mucin), melanoma associated antigen (MAGE), preferentially expressed antigen of melanoma (PRAME), carcinoembryonic antigen (CEA), prostate-specific antigen (PSA), prostate specific membrane antigen (PSMA), granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR), CD56, human epidermal growth factor receptor 2 (HER2/neu) (also known as erbB-2), CDS, CD7, tyrosinase tumour antigen, tyrosinase related protein (TRP) I, TRP2, NY-ESO-1, telomerase, and p53. In a preferred embodiment, the cell surface marker, to which the targeting moiety (cell-binding agent) specifically binds, is selected from the group consisting of cluster of differentiation (CD) 19, CD21, CD22, CD25, CD30, CD33 (sialic acid binding Ig-like lectin 3, myeloid cell surface antigen), CD79b, CD123 (interleukin 3 receptor alpha), transferrin receptor, EGF receptor, mesothelin, cadherin, Lewis Y, Glypican-3, FAP (fibroblast activation protein alpha), PSMA (prostate specific membrane antigen), CA9 = CAIX (carbonic anhydrase IX), L1 CAM (neural cell adhesion molecule L 1), endosialin, HER3 (activated conformation of epidermal growth factor receptor family member 3), Alkl/BMP9 complex (anaplastic lymphoma kinase 1/bone morphogenetic protein 9), TPBG = 5T4 (trophoblast glycoprotein), ROR1 (receptor tyrosine kinase-like surface antigen), HER1 (activated conformation of epidermal growth factor receptor), and CLL1 (C-type lectin domain family 12, member A). Mesothelin is expressed in, e.g., ovarian cancer, mesothelioma, non-small cell lung cancer, lung adenocarcinoma, fallopian tube cancer, head and neck cancer, cervical cancer, and pancreatic cancer. CD22 is expressed in, e.g., hairy cell leukemia, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), non-Hodgkin's lymphoma, small lymphocytic lymphoma (SLL), and acute lymphatic leukemia (ALL). CD25 is expressed in, e.g., leukemias and lymphomas, including hairy cell leukemia and Hodgkin's lymphoma. Lewis Y antigen is expressed in, e.g., bladder cancer, breast cancer, ovarian cancer, colorectal cancer, esophageal cancer, gastric cancer, lung cancer, and pancreatic cancer. CD33 is expressed in, e.g., acute myeloid leukemia (AML), chronic myelomonocytic leukemia (CML), and myeloproliferative disorders.

In an embodiment of the invention, the targeting moiety is an antibody that specifically binds to a tumour-associated antigen. Exemplary antibodies that specifically bind to tumour-associated antigens include, but are not limited to, antibodies against the transferrin receptor (e.g., HB21 and variants thereof), antibodies against CD22 (e.g., RFB4 and variants thereof), antibodies against CD25 (e.g., anti-Tac and variants thereof),

antibodies against mesothelin (e.g., SS 1, MORAb-009, SS, HN1, HN2, MN, MB, and variants thereof) and antibodies against Lewis Y antigen (e.g., B3 and variants thereof). In this regard, the targeting moiety (cell-binding agent) may be an antibody selected from the group consisting of B3, RFB4, SS, SS1, MN, MB, HN1, HN2, HB21, and MORAb-009, and antigen binding portions thereof. Further exemplary targeting moieties suitable for use in the inventive chimeric molecules are disclosed e.g., in U.S. Patents 5,242,824 (anti-transferrin receptor); 5,846,535 (anti-CD25); 5,889,157 (anti-Lewis Y); 5,981,726 (anti-Lewis Y); 5,990,296 (anti-Lewis Y); 7,081,518 (anti-mesothelin); 7,355,012 (anti-CD22 and anti-CD25); 7,368,110 (anti-mesothelin); 7,470,775 (anti-CD30); 7,521,054 (anti-CD25); and 7,541,034 (anti-CD22); U.S. Patent Application Publication 2007/0189962 (anti-CD22); Frankel et al., Clin. Cancer Res., 6: 326-334 (2000), and Kreitman et al., AAPS Journal, 8(3): E532-E551 (2006), each of which is incorporated herein by reference.

Antibodies have been raised to target specific tumour related antigens including: Cripto, CD30, CD19, CD33, Glycoprotein NMB, CanAg, Her2 (ErbB2/Neu), CD56 (NCAM), CD22 (Siglec2), CD33 (Siglec3), CD79, CD138, PSCA, PSMA (prostate specific membrane antigen), BCMA, CD20, CD70, E-selectin, EphB2, Melanotransferin, Muc16 and TMEFF2.

Labelled antibodies

In certain embodiments, the antibodies of the invention, in particular the anti-eEF2 antibodies of the invention, are labelled. Labels include, but are not limited to, labels or moieties that are detected directly (such as fluorescent, chromophoric, electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly, e.g., through an enzymatic reaction or molecular interaction. Exemplary labels include, but are not limited to, the radioisotopes ³²P, ¹⁴C, ¹²⁵I, ³H, and ¹³¹I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luciferases, e.g., firefly luciferase and bacterial luciferase (U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β -galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye

precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

Antibody Variants

- 5 In certain embodiments, amino acid sequence variants of the antibodies provided herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of an antibody may be prepared by introducing appropriate modifications into the nucleotide sequence encoding the antibody, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, e.g., antigen-binding.

a. Substitution, Insertion, and Deletion Variants

- In certain embodiments, antibody variants having one or more amino acid substitutions are provided. Sites of interest for substitutional mutagenesis include the HVRs and FRs. Conservative substitutions are shown in the table below under the heading of "conservative substitutions." More substantial changes are provided in Table 1 under the heading of "exemplary substitutions," and as further described below in reference to amino acid side chain classes. Amino acid substitutions may be introduced into an antibody of interest and the products screened for a desired activity, e.g., retained/improved antigen binding, decreased immunogenicity, or improved ADCC or CDC.

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

Amino acids may be grouped according to common side-chain properties:

- (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;
- (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;
- (3) acidic: Asp, Glu;
- (4) basic: His, Lys, Arg;
- (5) residues that influence chain orientation: Gly, Pro;
- (6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g. a humanized or human antibody). Generally, the resulting variant(s) selected for further study will have modifications (e.g., improvements) in certain biological properties (e.g., increased affinity, reduced immunogenicity) relative to the parent antibody and/or will have substantially retained certain biological properties of the parent antibody. An exemplary substitutional variant is an affinity matured antibody, which may be conveniently generated, e.g., using phage display-based affinity maturation techniques such as those described herein. Briefly, one or more HVR residues are mutated and the variant antibodies displayed on phage and screened for a particular biological activity (e.g. binding affinity).

Alterations (e.g., substitutions) may be made in HVRs, e.g., to improve antibody affinity. Such alterations may be made in HVR "hotspots," i.e., residues encoded by codons that undergo mutation at high frequency during the somatic maturation process (see, e.g., Chowdhury, *Methods Mol. Biol.* 207:179-196 (2008)), and/or SDRs (a-CDRs), with the resulting variant VH or VL being tested for binding affinity. Affinity maturation by constructing and reselecting from secondary libraries has been described, e.g., in Hoogenboom et al. in *Methods in Molecular Biology* 178:1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, (2001).) In some embodiments of affinity maturation, diversity is introduced into the variable genes chosen for maturation by any of a variety of methods (e.g., error-prone PCR, chain shuffling, or oligonucleotide-directed mutagenesis). A secondary library is then created. The library is then screened to identify any antibody variants with the desired affinity. Another method to introduce diversity involves HVR-directed approaches, in which several HVR residues (e.g., 4-6 residues at a time) are randomized. HVR residues involved in antigen binding may be specifically identified, e.g., using alanine scanning mutagenesis or modeling. CDR-H3 and CDR-L3 in particular are often targeted.

In certain embodiments, substitutions, insertions, or deletions may occur within one or more HVRs so long as such alterations do not substantially reduce the ability of the antibody to bind antigen. For example, conservative alterations (e.g., conservative substitutions as provided herein) that do not substantially reduce binding affinity may be made in HVRs. Such alterations may be outside of HVR "hotspots" or SDRs. In certain embodiments of the variant VH and VL sequences provided above, each HVR either is unaltered, or contains no more than one, two or three amino acid substitutions.

A useful method for identification of residues or regions of an antibody that may be targeted for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (1989) *Science*, 244:1081-1085. In this method, a residue or group of target residues (e.g., charged residues such as arg, asp, his, lys, and glu) are identified and replaced by a neutral or negatively charged amino acid (e.g., alanine or polyalanine) to determine whether the interaction of the antibody with antigen is affected. Further substitutions may be introduced at the amino acid locations demonstrating functional sensitivity to the initial substitutions. Alternatively, or additionally, a crystal structure of an

antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues may be targeted or eliminated as candidates for substitution. Variants may be screened to determine whether they contain the desired properties.

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Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

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b. Glycosylation variants

In certain embodiments, an antibody provided herein is altered to increase or decrease the extent to which the antibody is glycosylated. Addition or deletion of glycosylation sites to an antibody may be conveniently accomplished by altering the amino acid sequence such that one or more glycosylation sites is created or removed.

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Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. Native antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc region. See, e.g., Wright et al. *TIBTECH* 15:26-32 (1997). The oligosaccharide may include various carbohydrates, e.g., mannose, N-acetyl glucosamine (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the "stem" of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an antibody of the invention may be made in order to create antibody variants with certain improved properties.

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In one embodiment, antibody variants are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. For example, the amount of fucose in such antibody may be from 1% to 80%, from 1% to 65%, from 5% to 65% or from 20% to 40%. The amount of fucose is determined by calculating the average amount of fucose within the sugar chain at Asn297, relative to the sum of all glycostructures attached to Asn 297 (e. g. complex, hybrid and high mannose structures) as measured

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by MALDI-TOF mass spectrometry, as described in WO 2008/077546, for example. Asn297 refers to the asparagine residue located at about position 297 in the Fc region (Eu numbering of Fc region residues); however, Asn297 may also be located about ± 3 amino acids upstream or downstream of position 297, i.e., between positions 294 and 300, due to minor sequence variations in antibodies. Such fucosylation variants may have improved ADCC function. See, e.g., US Patent Publication Nos. US 2003/0157108 (Presta, L.); US 2004/0093621 (Kyowa Hakko Kogyo Co., Ltd). Examples of publications related to "defucosylated" or "fucose-deficient" antibody variants include: US 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/0115614; US 2002/0164328; US 2004/0093621; US 2004/0132140; US 2004/0110704; US 2004/0110282; US 2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO2005/053742; WO2002/031140; Okazaki et al. *J. Mol. Biol.* 336:1239-1249 (2004); Yamane-Ohnuki et al. *Biotech. Bioeng.* 87: 614 (2004). Examples of cell lines capable of producing defucosylated antibodies include Lec13 CHO cells deficient in protein fucosylation (Ripka et al. *Arch. Biochem. Biophys.* 249:533-545 (1986); US Pat Appl No US 2003/0157108 A1, Presta, L; and WO 2004/056312 A1, Adams et al., especially at Example 11), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, *FUT8*, knockout CHO cells (see, e.g., Yamane-Ohnuki et al. *Biotech. Bioeng.* 87: 614 (2004); Kanda, Y. et al., *Biotechnol. Bioeng.*, 94(4):680-688 (2006); and WO2003/085107).

Antibodies variants are further provided with bisected oligosaccharides, e.g., in which a biantennary oligosaccharide attached to the Fc region of the antibody is bisected by GlcNAc. Such antibody variants may have reduced fucosylation and/or improved ADCC function. Examples of such antibody variants are described, e.g., in WO 2003/011878 (Jean-Mairet et al.); US Patent No. 6,602,684 (Umana et al.); and US 2005/0123546 (Umana et al.). Antibody variants with at least one galactose residue in the oligosaccharide attached to the Fc region are also provided. Such antibody variants may have improved CDC function. Such antibody variants are described, e.g., in WO 1997/30087 (Patel et al.); WO 1998/58964 (Raju, S.); and WO 1999/22764 (Raju, S.).

c. Fc region variants

In certain embodiments, one or more amino acid modifications may be introduced into the Fc region of an antibody provided herein, thereby generating an Fc region variant. The Fc region variant may comprise a human Fc region sequence (e.g., a human IgG1, IgG2, IgG3 or IgG4 Fc region)

comprising an amino acid modification (e.g. a substitution) at one or more amino acid positions.

In certain embodiments, the invention contemplates an antibody variant that possesses some but not all effector functions, which make it a desirable candidate for applications in which the half life of the antibody *in vivo* is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. *In vitro* and/or *in vivo* cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express Fc(RIII only, whereas monocytes express Fc(RI, Fc(RII and Fc(RIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-492 (1991). Non-limiting examples of *in vitro* assays to assess ADCC activity of a molecule of interest is described in U.S. Patent No. 5,500,362 (see, e.g. Hellstrom, I. et al. *Proc. Nat'l Acad. Sci. USA* 83:7059-7063 (1986)) and Hellstrom, I et al., *Proc. Nat'l Acad. Sci. USA* 82:1499-1502 (1985); 5,821,337 (see Bruggemann, M. et al., *J. Exp. Med.* 166:1351-1361 (1987)). Alternatively, non-radioactive assays methods may be employed (see, for example, ACTI™ non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, CA; and CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, e.g., in a animal model such as that disclosed in Clynes et al. *Proc. Nat'l Acad. Sci. USA* 95:652-656 (1998). Clq binding assays may also be carried out to confirm that the antibody is unable to bind Clq and hence lacks CDC activity. See, e.g., Clq and C3c binding ELISA in WO 2006/029879 and WO 2005/100402. To assess complement activation, a CDC assay may be performed (see, for example, Gazzano-Santoro et al., *J. Immunol. Methods* 202:163 (1996); Cragg, M.S. et al., *Blood* 101:1045-1052 (2003); and Cragg, M.S. and M.J. Glennie, *Blood* 103:2738-2743 (2004)). FcRn binding and *in vivo* clearance/half life determinations can also be performed using methods known in the art (see, e.g., Petkova, S.B. et al., *Int'l. Immunol.* 18(12):1759-1769 (2006)).

Antibodies with reduced effector function include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Patent No. 6,737,056). Such Fc mutants include Fc mutants with

substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called "DANA" Fc mutant with substitution of residues 265 and 297 to alanine (US Patent No. 7,332,581).

5 Certain antibody variants with improved or diminished binding to FcRs are described. (See, e.g., U.S. Patent No. 6,737,056; WO 2004/056312, and Shields et al., *J. Biol. Chem.* 9(2): 6591-6604 (2001).)

10 In certain embodiments, an antibody variant comprises an Fc region with one or more amino acid substitutions which improve ADCC, e.g., substitutions at positions 298, 333, and/or 334 of the Fc region (EU numbering of residues).

15 In some embodiments, alterations are made in the Fc region that result in altered (*i.e.*, either improved or diminished) C1q binding and/or Complement Dependent Cytotoxicity (CDC), e.g., as described in US Patent No. 6,194,551, WO 99/51642, and Idusogie et al. *J. Immunol.* 164: 4178-4184 (2000).

20 Antibodies with increased half lives and improved binding to the neonatal Fc receptor (FcRn), which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)), are described in US2005/0014934A1 (Hinton et al.). Those antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. Such
25 Fc variants include those with substitutions at one or more of Fc region residues: 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434, e.g., substitution of Fc region residue 434 (US Patent No. 7,371,826).

30 See also Duncan & Winter, *Nature* 322:738-40 (1988); U.S. Patent No. 5,648,260; U.S. Patent No. 5,624,821; and WO 94/29351 concerning other examples of Fc region variants.

d. Cysteine engineered antibody variants

35 In certain embodiments, it may be desirable to create cysteine engineered antibodies, e.g., "thioMAbs," in which one or more residues of an antibody are substituted with cysteine residues. In particular embodiments, the substituted residues occur at accessible sites of the antibody. By
40 substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the antibody and may be used to

conjugate the antibody to other moieties, such as drug moieties or linker-drug moieties, to create an immunoconjugate, as described further herein. In certain embodiments, any one or more of the following residues may be substituted with cysteine: V205 (Kabat numbering) of the light chain; A118
5 (EU numbering) of the heavy chain; and S400 (EU numbering) of the heavy chain Fc region. Cysteine engineered antibodies may be generated as described, e.g., in U.S. Patent No. 7,521,541.

e. Antibody Derivatives

10 In certain embodiments, an antibody provided herein may be further modified to contain additional nonproteinaceous moieties that are known in the art and readily available. The moieties suitable for derivatization of the antibody include but are not limited to water soluble polymers. Non-
15 limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random
20 copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide copolymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any
25 molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer is attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular
30 properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, etc.

In another embodiment, conjugates of an antibody and nonproteinaceous moiety that may be selectively heated by exposure to radiation are
35 provided. In one embodiment, the nonproteinaceous moiety is a carbon nanotube (Kam et al., *Proc. Natl. Acad. Sci. USA* 102: 11600-11605 (2005)). The radiation may be of any wavelength, and includes, but is not limited to, wavelengths that do not harm ordinary cells, but which heat the
40 nonproteinaceous moiety to a temperature at which cells proximal to the antibody-nonproteinaceous moiety are killed.

Antibody Fragments

In certain embodiments, an antibody provided herein is an antibody
5 fragment. Antibody fragments include, but are not limited to, Fab, Fab',
Fab'-SH, F(ab')₂, Fv, and scFv fragments, and other fragments described
below. For a review of certain antibody fragments, see Hudson et al. *Nat.*
Med. 9:129-134 (2003). For a review of scFv fragments, see, e.g.,
10 Pluckthün, in *The Pharmacology of Monoclonal Antibodies*, vol. 113,
Rosenburg and Moore eds., (Springer-Verlag, New York), pp. 269-315 (1994);
see also WO 93/16185; and U.S. Patent Nos. 5,571,894 and 5,587,458. For
discussion of Fab and F(ab')₂ fragments comprising salvage receptor binding
epitope residues and having increased in vivo half-life, see U.S. Patent
15 No. 5,869,046.

Diabodies are antibody fragments with two antigen-binding sites that may be
bivalent or bispecific. See, for example, EP 404,097; WO 1993/01161;
Hudson et al., *Nat. Med.* 9:129-134 (2003); and Hollinger et al., *Proc.*
Natl. Acad. Sci. USA 90: 6444-6448 (1993). Triabodies and tetrabodies are
20 also described in Hudson et al., *Nat. Med.* 9:129-134 (2003).

Single-domain antibodies are antibody fragments comprising all or a portion
of the heavy chain variable domain or all or a portion of the light chain
variable domain of an antibody. In certain embodiments, a single-domain
25 antibody is a human single-domain antibody (Domantis, Inc., Waltham, MA;
see, e.g., U.S. Patent No. 6,248,516 B1).

Antibody fragments can be made by various techniques, including but not
limited to proteolytic digestion of an intact antibody as well as
30 production by recombinant host cells (e.g. *E. coli* or phage), as described
herein.

Chimeric and Humanized Antibodies

In certain embodiments, an antibody provided herein (in particular, an
35 antibody provided as a cell-binding agent) is a chimeric antibody. Certain
chimeric antibodies are described, e.g., in U.S. Patent No. 4,816,567; and
Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)). In one
example, a chimeric antibody comprises a non-human variable region (e.g., a
variable region derived from a mouse, rat, hamster, rabbit, or non-human
40 primate, such as a monkey) and a human constant region. In a further
example, a chimeric antibody is a "class switched" antibody in which the

class or subclass has been changed from that of the parent antibody.
Chimeric antibodies include antigen-binding fragments thereof.

In certain embodiments, a chimeric antibody is a humanized antibody.

5 Typically, a non-human antibody is humanized to reduce immunogenicity to humans, while retaining the specificity and affinity of the parental non-human antibody. Generally, a humanized antibody comprises one or more variable domains in which HVRs, e.g., CDRs, (or portions thereof) are derived from a non-human antibody, and FRs (or portions thereof) are
10 derived from human antibody sequences. A humanized antibody optionally will also comprise at least a portion of a human constant region. In some embodiments, some FR residues in a humanized antibody are substituted with corresponding residues from a non-human antibody (e.g., the antibody from which the HVR residues are derived), e.g., to restore or improve antibody
15 specificity or affinity.

Humanized antibodies and methods of making them are reviewed, e.g., in Almagro and Fransson, *Front. Biosci.* 13:1619-1633 (2008), and are further described, e.g., in Riechmann et al., *Nature* 332:323-329 (1988); Queen et al., *Proc. Nat'l Acad. Sci. USA* 86:10029-10033 (1989); US Patent Nos. 5, 821,337, 7,527,791, 6,982,321, and 7,087,409; Kashmiri et al., *Methods* 36:25-34 (2005) (describing SDR (a-CDR) grafting); Padlan, *Mol. Immunol.* 28:489-498 (1991) (describing "resurfacing"); Dall'Acqua et al., *Methods* 36:43-60 (2005) (describing "FR shuffling"); and Osbourn et al., *Methods* 36:61-68 (2005) and Klimka et al., *Br. J. Cancer*, 83:252-260 (2000) (describing the "guided selection" approach to FR shuffling).

Human framework regions that may be used for humanization include but are not limited to: framework regions selected using the "best-fit" method
30 (see, e.g., Sims et al. *J. Immunol.* 151:2296 (1993)); framework regions derived from the consensus sequence of human antibodies of a particular subgroup of light or heavy chain variable regions (see, e.g., Carter et al. *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); and Presta et al. *J. Immunol.*, 151:2623 (1993)); human mature (somatically mutated) framework regions or
35 human germline framework regions (see, e.g., Almagro and Fransson, *Front. Biosci.* 13:1619-1633 (2008)); and framework regions derived from screening FR libraries (see, e.g., Baca et al., *J. Biol. Chem.* 272:10678-10684 (1997) and Rosok et al., *J. Biol. Chem.* 271:22611-22618 (1996)).

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Human Antibodies

In certain embodiments, an antibody provided herein (in particular, an antibody provided as a cell-binding agent) is a human antibody. Human antibodies can be produced using various techniques known in the art.

5 Human antibodies are described generally in van Dijk and van de Winkel, *Curr. Opin. Pharmacol.* 5: 368-74 (2001) and Lonberg, *Curr. Opin. Immunol.* 20:450-459 (2008).

10 Human antibodies may be prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions in response to antigenic challenge. Such animals typically contain all or a portion of the human immunoglobulin loci, which replace the endogenous immunoglobulin loci, or which are present extrachromosomally or integrated randomly into the
15 animal's chromosomes. In such transgenic mice, the endogenous immunoglobulin loci have generally been inactivated. For review of methods for obtaining human antibodies from transgenic animals, see Lonberg, *Nat. Biotech.* 23:1117-1125 (2005). See also, e.g., U.S. Patent Nos. 6,075,181 and 6,150,584 describing XENOMOUSE™ technology; U.S. Patent No. 5,770,429
20 describing HuMab® technology; U.S. Patent No. 7,041,870 describing K-M MOUSE® technology, and U.S. Patent Application Publication No. US 2007/0061900, describing VelociMouse® technology). Human variable regions from intact antibodies generated by such animals may be further modified, e.g., by combining with a different human constant region.

25 Human antibodies can also be made by hybridoma-based methods. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described. (See, e.g., Kozbor *J. Immunol.*, 133: 3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner et al., *J. Immunol.*, 147: 86 (1991).) Human antibodies
30 generated via human B-cell hybridoma technology are also described in Li et al., *Proc. Natl. Acad. Sci. USA*, 103:3557-3562 (2006). Additional methods include those described, for example, in U.S. Patent No. 7,189,826
35 (describing production of monoclonal human IgM antibodies from hybridoma cell lines) and Ni, *Xiandai Mianyixue*, 26(4):265-268 (2006) (describing human-human hybridomas). Human hybridoma technology (Trioma technology) is also described in Vollmers and Brandlein, *Histology and Histopathology*, 20(3):927-937 (2005) and Vollmers and Brandlein, *Methods and Findings in Experimental and Clinical Pharmacology*, 27(3):185-91 (2005).
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Human antibodies may also be generated by isolating Fv clone variable domain sequences selected from human-derived phage display libraries. Such variable domain sequences may then be combined with a desired human constant domain. Techniques for selecting human antibodies from antibody libraries are described below.

Library-derived antibodies

Antibodies of the invention may be isolated by screening combinatorial libraries for antibodies with the desired activity or activities. For example, a variety of methods are known in the art for generating phage display libraries and screening such libraries for antibodies possessing the desired binding characteristics. Such methods are reviewed, e.g., in Hoogenboom et al. in *Methods in Molecular Biology* 178:1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, 2001) and further described, e.g., in the McCafferty et al., *Nature* 348:552-554; Clackson et al., *Nature* 352: 624-628 (1991); Marks et al., *J. Mol. Biol.* 222: 581-597 (1992); Marks and Bradbury, in *Methods in Molecular Biology* 248:161-175 (Lo, ed., Human Press, Totowa, NJ, 2003); Sidhu et al., *J. Mol. Biol.* 338(2): 299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34): 12467-12472 (2004); and Lee et al., *J. Immunol. Methods* 284(1-2): 119-132(2004).

In certain phage display methods, repertoires of VH and VL genes are separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be screened for antigen-binding phage as described in Winter et al., *Ann. Rev. Immunol.*, 12: 433-455 (1994). Phage typically display antibody fragments, either as single-chain Fv (scFv) fragments or as Fab fragments. Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned (e.g., from human) to provide a single source of antibodies to a wide range of non-self and also self antigens without any immunization as described by Griffiths et al., *EMBO J*, 12: 725-734 (1993). Finally, naive libraries can also be made synthetically by cloning unrearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement *in vitro*, as described by Hoogenboom and Winter, *J. Mol. Biol.*, 227: 381-388 (1992). Patent publications describing human antibody phage libraries include, for example: US Patent No. 5,750,373, and US Patent Publication Nos. 2005/0079574, 2005/0119455, 2005/0266000, 2007/0117126, 2007/0160598, 2007/0237764, 2007/0292936, and 2009/0002360.

Antibodies or antibody fragments isolated from human antibody libraries are considered human antibodies or human antibody fragments herein.

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Multispecific Antibodies

In certain embodiments, an antibody provided herein is a multispecific antibody, e.g. a bispecific antibody. Multispecific antibodies are monoclonal antibodies that have binding specificities for at least two
10 different sites. In certain embodiments, one of the binding specificities is for linear linked polyubiquitin and the other is for any other antigen. In certain embodiments, bispecific antibodies may bind to two different epitopes of linear linked polyubiquitin. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express linear linked
15 polyubiquitin. Bispecific antibodies can be prepared as full length antibodies or antibody fragments.

Techniques for making multispecific antibodies include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain
20 pairs having different specificities (see Milstein and Cuello, *Nature* 305: 537 (1983)), WO 93/08829, and Traunecker et al., *EMBO J.* 10: 3655 (1991)), and "knob-in-hole" engineering (see, e.g., U.S. Patent No. 5,731,168). Multi-specific antibodies may also be made by engineering electrostatic steering effects for making antibody Fc-heterodimeric molecules
25 (WO 2009/089004A1); cross-linking two or more antibodies or fragments (see, e.g., US Patent No. 4,676,980, and Brennan et al., *Science*, 229: 81 (1985)); using leucine zippers to produce bi-specific antibodies (see, e.g., Kostelny et al., *J. Immunol.*, 148(5):1547-1553 (1992)); using "diabody" technology for making bispecific antibody fragments (see, e.g.,
30 Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993)); and using single-chain Fv (sFv) dimers (see, e.g. Gruber et al., *J. Immunol.*, 152:5368 (1994)); and preparing trispecific antibodies as described, e.g., in Tutt et al. *J. Immunol.* 147: 60 (1991).

35 Engineered antibodies with three or more functional antigen binding sites, including "Octopus antibodies," are also included herein (see, e.g. US 2006/0025576A1).

The antibody or fragment herein also includes a "Dual Acting FAb" or "DAF"
40 comprising an antigen binding site that binds to linear linked polyubiquitin as well as another, different antigen (see, US 2008/0069820, for example).

Recombinant Methods and Compositions

Targeted therapeutic agents of the invention that comprise a NAD(+)-diphthamide ADP-ribosyltransferase coupled to a cell-binding agent (in
5 particular those coupled by fusion) may be obtained, for example, by solid-state peptide synthesis (e.g. Merrifield solid phase synthesis) or recombinant production. For recombinant production, one or more polynucleotides together encoding the targeted therapeutic agent are isolated and inserted into one or more vectors for further cloning and/or
10 expression in a host cell. Such polynucleotide may be readily isolated and sequenced using conventional procedures.

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the coding sequence(s) of the
15 targeted therapeutic agent along with appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. See, for example, the techniques described in Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold
20 Spring Harbor Laboratory, N.Y. (1989); and Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, N.Y (1989). The expression vector can be part of a plasmid, virus, or may be a nucleic acid fragment. The expression vector includes an expression cassette into which the polynucleotide encoding the targeted
25 therapeutic agent (i.e. the coding region) is cloned in operable association with a promoter and/or other transcription or translation control elements.

As used herein, a "coding region" is a portion of nucleic acid which
30 consists of codons translated into amino acids. Although a "stop codon" (TAG, TGA, or TAA) is not translated into an amino acid, it may be considered to be part of a coding region, if present, but any flanking sequences, for example promoters, ribosome binding sites, transcriptional terminators, introns, 5' and 3' untranslated regions, and the like, are not
35 part of a coding region. Two or more coding regions can be present in a single polynucleotide construct, e.g. on a single vector, or in separate polynucleotide constructs, e.g. on separate (different) vectors.

Furthermore, any vector may contain a single coding region, or may comprise two or more coding regions, e.g. a vector of the present invention may
40 encode one or more polypeptides, which are post- or co-translationally separated into the final protein via proteolytic cleavage (for example in

the case of targeted therapeutic agents in which the NAD(+)-diphthamide ADP ribosyltransferase is coupled to the cell-binding agent via a disulphide bond, rather than as a fusion polypeptide via a peptide bond). In addition, a vector, polynucleotide, or nucleic acid of the invention may encode

5 heterologous coding regions, either fused or unfused to a polynucleotide encoding the targeted therapeutic agent of the invention, or variant or derivative thereof. Heterologous coding regions include without limitation specialized elements or motifs, such as a secretory signal peptide or a heterologous functional domain. An operable association is when a coding

10 region for a gene product, e.g. a polypeptide, is associated with one or more regulatory sequences in such a way as to place expression of the gene product under the influence or control of the regulatory sequence(s). Two DNA fragments (such as a polypeptide coding region and a promoter associated therewith) are "operably associated" if induction of promoter

15 function results in the transcription of mRNA encoding the desired gene product and if the nature of the linkage between the two DNA fragments does not interfere with the ability of the expression regulatory sequences to direct the expression of the gene product or interfere with the ability of the DNA template to be transcribed. Thus, a promoter region would be

20 operably associated with a nucleic acid encoding a polypeptide if the promoter was capable of effecting transcription of that nucleic acid. The promoter may be a cell-specific promoter that directs substantial transcription of the DNA only in predetermined cells. Other transcription control elements, besides a promoter, for example enhancers, operators,

25 repressors, and transcription termination signals, can be operably associated with the polynucleotide to direct cell-specific transcription. Suitable promoters and other transcription control regions are disclosed herein. A variety of transcription control regions are known to those skilled in the art. These include, without limitation, transcription

30 control regions, which function in vertebrate cells, such as, but not limited to, promoter and enhancer segments from cytomegaloviruses (e.g. the immediate early promoter, in conjunction with intron-A), simian virus 40 (e.g. the early promoter), and retroviruses (such as, e.g. Rous sarcoma virus). Other transcription control regions include those derived from

35 vertebrate genes such as actin, heat shock protein, bovine growth hormone and rabbit α -globin, as well as other sequences capable of controlling gene expression in eukaryotic cells. Additional suitable transcription control regions include tissue-specific promoters and enhancers as well as inducible promoters (e.g. promoters inducible tetracyclins). Similarly, a

40 variety of translation control elements are known to those of ordinary skill in the art. These include, but are not limited to ribosome binding

sites, translation initiation and termination codons, and elements derived from viral systems (particularly an internal ribosome entry site, or IRES, also referred to as a CITE sequence). The expression cassette may also include other features such as an origin of replication, and/or chromosome
5 integration elements such as retroviral long terminal repeats (LTRs), or adeno-associated viral (AAV) inverted terminal repeats (ITRs).

Polynucleotide and nucleic acid coding regions may be associated with additional coding regions which encode secretory or signal peptides, which
10 direct the secretion of the targeted therapeutic agent of the present invention. For example, if secretion of the targeted therapeutic agent is desired, DNA encoding a signal sequence may be placed upstream of the nucleic acid encoding the targeted therapeutic agent of the invention.

According to the signal hypothesis, proteins secreted by mammalian cells
15 have a signal peptide or secretory leader sequence which is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Those of ordinary skill in the art are aware that polypeptides secreted by vertebrate cells generally have a signal peptide fused to the N-terminus of the polypeptide, which is
20 cleaved from the translated polypeptide to produce a secreted or "mature" form of the polypeptide. In certain embodiments, the native signal peptide, e.g. an immunoglobulin heavy chain or light chain signal peptide is used, or a functional derivative of that sequence that retains the ability to direct the secretion of the polypeptide that is operably associated with
25 it. Alternatively, a heterologous mammalian signal peptide, or a functional derivative thereof, may be used. For example, the wild-type leader sequence may be substituted with the leader sequence of human tissue plasminogen activator (TPA) or mouse β -glucuronidase.

30 DNA encoding a short protein sequence that could be used to facilitate later purification (e.g. a histidine tag) or assist in labeling the targeted therapeutic agent may be included within or at the ends of the targeted therapeutic agent.

35 As used herein, the term "host cell" refers to any kind of cellular system which can be engineered to generate the targeted therapeutic agent of the invention. Host cells suitable for replicating and for supporting expression of the targeted therapeutic agents of the invention are well known in the art. Such cells may be transfected or transduced as
40 appropriate with the particular expression vector and large quantities of vector containing cells can be grown for seeding large scale fermenters to

obtain sufficient quantities of the targeted therapeutic agent for clinical applications. Suitable host cells include prokaryotic microorganisms, such as *E. coli*, or various eukaryotic cells, such as Chinese hamster ovary cells (CHO), insect cells, or the like. For example, polypeptides may be produced in bacteria in particular when glycosylation is not needed. After expression, the polypeptide may be isolated from the bacterial cell paste in a soluble fraction and can be further purified. In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been "humanized", resulting in the production of a polypeptide with a partially or fully human glycosylation pattern. See Gerngross, *Nat Biotech* 22, 1409-1414 (2004), and Li et al., *Nat Biotech* 24, 210-215 (2006). Suitable host cells for the expression of (glycosylated) polypeptides are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains have been identified which may be used in conjunction with insect cells, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures can also be utilized as hosts. See e.g. US Patent Nos. 5,959,177, 6,040,498, 6,420,548, 7,125,978, and 6,417,429 (describing PLANTIBODIES™ technology for producing antibodies in transgenic plants). Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. Other examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293T cells as described, e.g., in Graham et al., *J Gen Virol* 36, 59 (1977)), baby hamster kidney cells (BHK), mouse sertoli cells (TM4 cells as described, e.g., in Mather, *Biol Reprod* 23, 243-251 (1980)), monkey kidney cells (CV1), African green monkey kidney cells (VERO-76), human cervical carcinoma cells (HELA), canine kidney cells (MDCK), buffalo rat liver cells (BRL 3A), human lung cells (W138), human liver cells (Hep G2), mouse mammary tumour cells (MMT 060562), TRI cells (as described, e.g., in Mather et al., *Annals N.Y. Acad Sci* 383, 44-68 (1982)), MRC 5 cells, and FS4 cells. Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including dhfr- CHO cells (Urlaub et al., *Proc Natl Acad Sci USA* 77, 4216 (1980)); and myeloma cell lines such as YO, NS0, P3X63 and Sp2/0. For a review of certain mammalian host cell lines suitable for protein production, see, e.g., Yazaki and Wu, *Methods in Molecular Biology*, Vol. 248 (B.K.C. Lo, ed., Humana Press, Totowa, NJ), pp. 255-268 (2003). Host cells include cultured cells, e.g., mammalian cultured cells, yeast cells, insect cells, bacterial cells and plant cells, to name only a few, but also cells

comprised within a transgenic animal, transgenic plant or cultured plant or animal tissue. In one embodiment, the host cell is a eukaryotic cell, preferably a mammalian cell, such as a Chinese Hamster Ovary (CHO) cell, a human embryonic kidney (HEK) cell or a lymphoid cell (e.g., YO, NS0, Sp20 cell). Typically, the NAD(+)-diphthamide ADP ribosyltransferases of the invention are produced in prokaryotic cells, such as E. coli.

Standard technologies are known in the art to express foreign genes in these systems. Cells expressing a polypeptide comprising either the heavy or the light chain of an antigen binding domain such as an antibody, may be engineered so as to also express the other of the antibody chains fused to a NAD(+)-diphthamide ADP ribosyltransferase such that the expressed product is an antibody that has both a heavy and a light chain.

A method of producing a targeted therapeutic agent according to the invention may comprise culturing a host cell comprising a polynucleotide encoding the targeted therapeutic agent under conditions suitable for expression of the targeted therapeutic agent, and recovering the targeted therapeutic agent from the host cell (or host cell culture medium).

The components of the targeted therapeutic agent may be genetically fused to each other. The targeted therapeutic agent can be designed such that its components are fused directly to each other or indirectly through a linker sequence. The composition and length of the linker may be determined in accordance with methods well known in the art and may be tested for efficacy.

In certain embodiments the Fab fragments forming part of the targeted therapeutic agent comprise at least an antibody variable region capable of binding an antigenic determinant. Variable regions can form part of and be derived from naturally or non-naturally occurring antibodies and fragments thereof. Methods to produce polyclonal antibodies and monoclonal antibodies are well known in the art (see e.g. Harlow and Lane, "Antibodies, a laboratory manual", Cold Spring Harbor Laboratory, 1988). Non-naturally occurring antibodies can be constructed using solid phase-peptide synthesis, can be produced recombinantly (e.g. as described in U.S. patent No. 4,186,567) or can be obtained, for example, by screening combinatorial libraries comprising variable heavy chains and variable light chains (see e.g. U.S. Patent. No. 5,969,108 to McCafferty).

Any animal species of antibody, antibody fragment, antigen binding domain or variable region can be used in the targeted therapeutic agents of the invention. Non-limiting antibodies, antibody fragments, antigen binding domains or variable regions useful in the present invention can be of murine, primate, or human origin. If the targeted therapeutic agent is intended for human use, a chimeric form of antibody may be used wherein the constant regions of the antibody are from a human. A humanized or fully human form of the antibody can also be prepared in accordance with methods well known in the art (see e. g. U.S. Patent No. 5,565,332 to Winter).

Humanization may be achieved by various methods including, but not limited to (a) grafting the non-human (e.g., donor antibody) CDRs onto human (e.g. recipient antibody) framework and constant regions with or without retention of critical framework residues (e.g. those that are important for retaining good antigen binding affinity or antibody functions), (b) grafting only the non-human specificity-determining regions (SDRs or a-CDRs; the residues critical for the antibody-antigen interaction) onto human framework and constant regions, or (c) transplanting the entire non-human variable domains, but "cloaking" them with a human-like section by replacement of surface residues. Humanized antibodies and methods of making them are reviewed, e.g., in Almagro and Fransson, *Front Biosci* 13, 1619-1633 (2008), and are further described, e.g., in Riechmann et al., *Nature* 332, 323-329 (1988); Queen et al., *Proc Natl Acad Sci USA* 86, 10029-10033 (1989); US Patent Nos. 5,821,337, 7,527,791, 6,982,321, and 7,087,409; Jones et al., *Nature* 321, 522-525 (1986); Morrison et al., *Proc Natl Acad Sci* 81, 6851-6855 (1984); Morrison and Oi, *Adv Immunol* 44, 65-92 (1988); Verhoeven et al., *Science* 239, 1534-1536 (1988); Padlan, *Molec Immun* 31(3), 169-217 (1994); Kashmiri et al., *Methods* 36, 25-34 (2005) (describing SDR (a-CDR) grafting); Padlan, *Mol Immunol* 28, 489-498 (1991) (describing "resurfacing"); Dall'Acqua et al., *Methods* 36, 43-60 (2005) (describing "FR shuffling"); and Osbourn et al., *Methods* 36, 61-68 (2005) and Klimka et al., *Br J Cancer* 83, 252-260 (2000) (describing the "guided selection" approach to FR shuffling). Human antibodies and human variable regions can be produced using various techniques known in the art. Human antibodies are described generally in van Dijk and van de Winkel, *Curr Opin Pharmacol* 5, 368-74 (2001) and Lonberg, *Curr Opin Immunol* 20, 450-459 (2008). Human variable regions can form part of and be derived from human monoclonal antibodies made by the hybridoma method (see e.g. *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)). Human antibodies and human variable regions may also be prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human

variable regions in response to antigenic challenge (see e.g. Lonberg, Nat Biotech 23, 1117-1125 (2005). Human antibodies and human variable regions may also be generated by isolating Fv clone variable region sequences selected from human-derived phage display libraries (see e.g., Hoogenboom et al. in Methods in Molecular Biology 178, 1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, 2001); and McCafferty et al., Nature 348, 552-554; Clackson et al., Nature 352, 624-628 (1991)). Phage typically display antibody fragments, either as single-chain Fv (scFv) fragments or as Fab fragments.

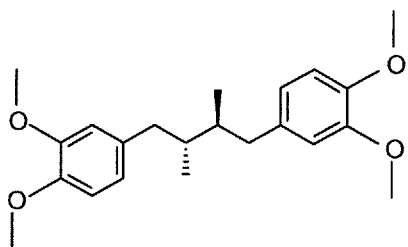
In certain embodiments, the Fab fragments useful in the present invention are engineered to have enhanced binding affinity according to, for example, the methods disclosed in U.S. Pat. Appl. Publ. No. 2004/0132066, the entire contents of which are hereby incorporated by reference. The ability of the targeted therapeutic agent of the invention to bind to a specific antigenic determinant can be measured either through an enzyme-linked immunosorbent assay (ELISA) or other techniques familiar to one of skill in the art, e.g. surface plasmon resonance technique (analyzed on a BIACORE T100 system) (Liljeblad, et al., Glyco J 17, 323-329 (2000)), and traditional binding assays (Heeley, Endocr Res 28, 217-229 (2002)). Competition assays may be used to identify an antibody, antibody fragment, antigen binding domain or variable domain that competes with a reference antibody for binding to a particular antigen. In certain embodiments, such a competing antibody binds to the same epitope (e.g. a linear or a conformational epitope) that is bound by the reference antibody. Detailed exemplary methods for mapping an epitope to which an antibody binds are provided in Morris (1996) "Epitope Mapping Protocols," in Methods in Molecular Biology vol. 66 (Humana Press, Totowa, NJ). In an exemplary competition assay, immobilized antigen is incubated in a solution comprising a first labeled antibody that binds to the antigen and a second unlabeled antibody that is being tested for its ability to compete with the first antibody for binding to the antigen. The second antibody may be present in a hybridoma supernatant. As a control, immobilized antigen is incubated in a solution comprising the first labeled antibody but not the second unlabeled antibody. After incubation under conditions permissive for binding of the first antibody to the antigen, excess unbound antibody is removed, and the amount of label associated with immobilized antigen is measured. If the amount of label associated with immobilized antigen is substantially reduced in the test sample relative to the control sample, then that indicates that the second antibody is competing with the first antibody for binding to the antigen. See Harlow

and Lane (1988) Antibodies: A Laboratory Manual ch.14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

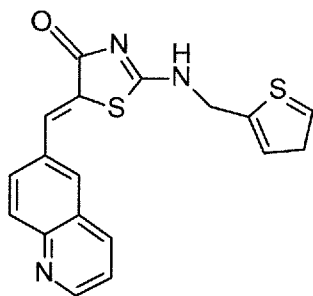
Targeted therapeutic agents prepared as described herein may be purified by art-known techniques such as high performance liquid chromatography, ion exchange chromatography, gel electrophoresis, affinity chromatography, size exclusion chromatography, and the like. The actual conditions used to purify a particular protein will depend, in part, on factors such as net charge, hydrophobicity, hydrophilicity etc., and will be apparent to those having skill in the art. For affinity chromatography purification an antibody, ligand, receptor or antigen can be used to which the targeted therapeutic agent binds. For example, for affinity chromatography purification targeted therapeutic agents that include antibody sequences as cell-binding agents, a matrix with protein A or protein G may be used. Sequential Protein A or G affinity chromatography and size exclusion chromatography can be used to isolate the targeted therapeutic agent. The purity of the targeted therapeutic agent can be determined by any of a variety of well known analytical methods including gel electrophoresis, high pressure liquid chromatography, and the like.

Exemplary CDK1 inhibitors

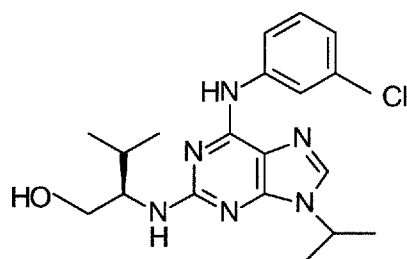
Terameprocol (EM-1421, Erimos Pharmaceuticals) is a transcriptional inhibitor that competes with the transcription factor specificity protein 1 (Sp1) (Smolewski, 2008). This agent suppresses transcription of the Sp1-dependant genes CDK1, survivin and vascular endothelial growth factor (VEGF) and is currently undergoing clinical investigation. In chemical terms, terameprocol is named 4-[4-(3,4-dimethoxyphenyl)-2,3-dimethylbutyl]-1,2-dimethoxybenzene and has the structure:



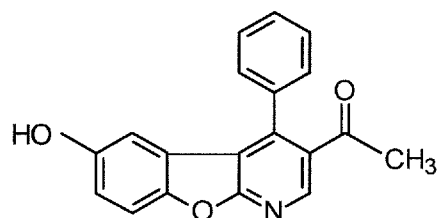
RO-3306 (Roche) is an ATP competitive inhibitor, which selectively inhibits CDK1 activity (Vassilev et al., 2006). In chemical terms, RO-3306 is named 5-(quinolin-6-ylmethylidene)-2-(thiophen-2-ylmethylamino)-1,3-thiazol-4-one and has the structure:



Purvalanol A is a CDK inhibitor with high selectivity for CDK1 and lower selectivity for CDK2 and CDK5 (Gray et al., 1998). In chemical terms, Purvalanol A is named (2R)-2-({6-[(3-chlorophenyl)amino]-9-(propan-2-yl)-5H-purin-2-yl}amino)-3-methylbutan-1-ol and has the structure:

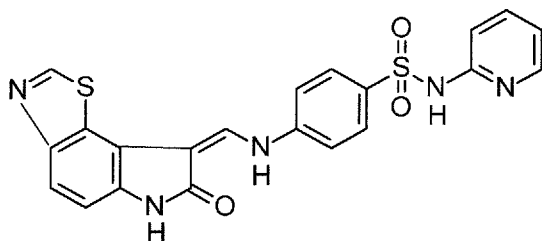


Elbfluorene (Enzo Life Sciences) is a potent inhibitor of CDK1 that weakly inhibits CDK5 (Voigt et al., 2005). In chemical terms it is named 3-Acetyl-6-hydroxy-4-phenylbenzo[4,5]furo[2,3-b]pyridine, 1-Aza-9-oxafluorene 5b and has the structure:

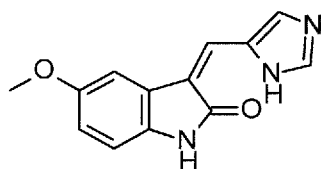


Exemplary CDK2 inhibitors

GW8510 (GlaxoSmithKline) is a 3' substituted indolone that was developed as a selective CDK2 inhibitor (Davis et al., 2001) and later found to have activity against CDK1, CDK3 and CDK5 (Johnson et al., 2005). In chemical terms, GW8510 is named 4-{[(7-Oxo-6,7-dihydro-8H-[1,3]thiazolo[5,4-e]indol-8-ylidene)methyl]amino}-N-(2-pyridyl)benzenesulfonamide and has the structure:

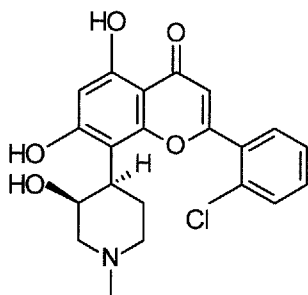


SU9516 (SUGEN) is a potent CDK inhibitor with high selectivity for CDK2 and lower selectivity for CDK4 and CDK1 (Lane et al., 2001). In chemical terms, SU9516 is named (3Z)-3-(1H-imidazol-5-ylmethylidene)-5-methoxy-1H-indol-2-one and has the structure:

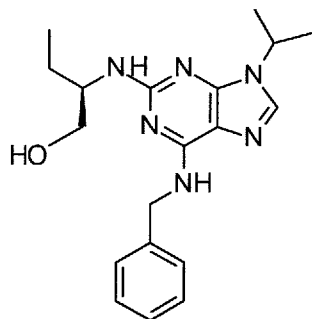


Exemplary pan-CDK inhibitors

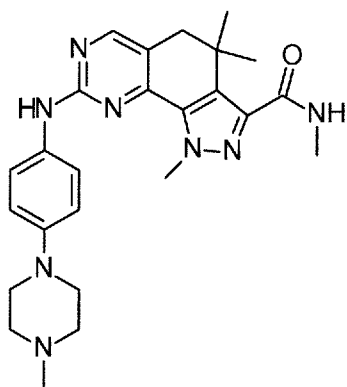
Alvocidib (Flavopiridol or HMR-1275, Sanofi) is a flavonoid derived from an Indian plant, rohitukine, that functions as an ATP-competitive inhibitor against CDK1, CDK2, CDK4, CDK6, CDK7 and CDK9 (Sedlacek et al., 1996). The therapeutic use of alvocidib is being investigated in clinical trials. Alvocidib is named 2-(2-chlorophenyl)-5,7-dihydroxy-8-[(3S,4R)-3-hydroxy-1-methyl-4-piperidinyl]-4-chromenone and has the structure:



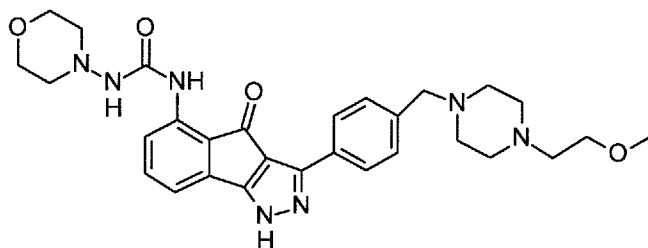
Seliciclib ((R)-Roscovitine or CYC202, Cyclacel) is a competitive inhibitor for ATP that selectively inhibits CDK1, CDK2, CDK5, CDK7 and CDK9 (Meijer et al., 1997; Wang et al., 2001). Seliciclib is currently being evaluated in clinical trials. In chemical terms it is named 2-(R)-(1-Ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine and has the structure:



Milciclib (PHA-848125, Nerviano Medical Sciences) is a potent inhibitor of CDK1, CDK2, CDK4 and CDK5 and other receptor tyrosine kinases (Brasca et al., 2009). Milciclib is being evaluated in clinical trials. In chemical terms, it is named N,1,4,4-tetramethyl-8-((4-(4-methylpiperazin-1-yl)phenyl)amino)-4,5-dihydro-1H-pyrazolo[4,3-h]quinazoline-3-carboxamide and has the structure:

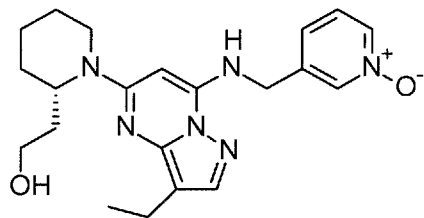


RGB-286638 (Agennix) is a multi-targeted inhibitor with activity against CDK proteins as well as a range of other cancer-relevant tyrosine and serine/threonine kinases (Cirstea et al., 2013). The use of RGB-286638 is being evaluated in clinical trials. In chemical terms it is named 1-[3-[4-[[4-(2-methoxyethyl)piperazin-1-yl]methyl]phenyl]-4-oxo-1H-indeno[1,2-c]pyrazol-5-yl]-3-morpholin-4-ylurea and has the structure:

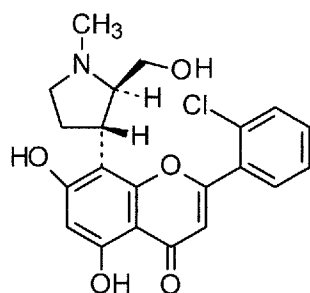


Dinaciclib (SCH 727965, Merck) is a broad-range CDK inhibitor with activity against CDK2, CDK5, CDK1 and CDK9 currently being evaluated in clinical trials (Paruch et al., 2010). In chemical terms, Dinaciclib is named (S)-

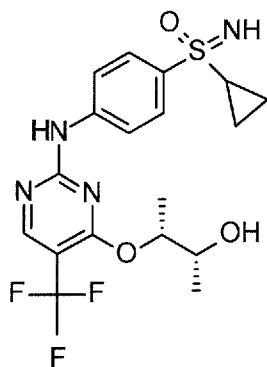
3-(((3-Ethyl-5-(2-(2-hydroxyethyl)piperidin-1-yl)pyrazolo[1,5-a]pyrimidin-7-yl)amino)methyl)pyridine 1-oxide and has the structure:



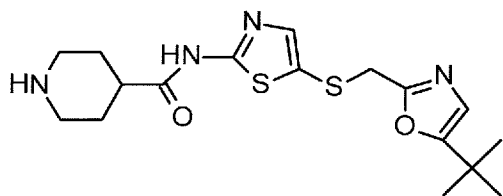
- 5 Riviciclib (P276-00, Nicholas Piramal) is an analogue of flavopiridol that selectively inhibits CDK4, CDK1 and CDK9 (Joshi et al., 2007). Use of riviciclib is being evaluated in clinical trials. In chemical terms it is named 2-(2-chlorophenyl)-5,7-dihydroxy-8-((2R,3S)-2-(hydroxymethyl)-1-methylpyrrolidin-3-yl)-4H-chromen-4-one and has the structure:



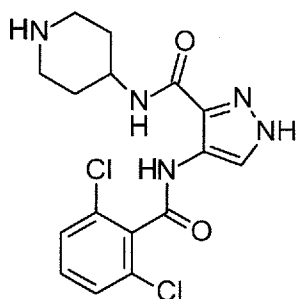
- 10 Roniciclib (BAY 1000394, Bayer) has been shown to inhibit the activity of CDK1, CDK2, CDK3, CD4, CDK7 and CDK9 and is being evaluated in clinical trials (Siemeister et al., 2012). In chemical terms, it is named (2R,3R)-3-((2-((4-(cyclopropanesulfonimidoyl)phenyl)amino)-5-(trifluoromethyl)pyrimidin-4-yl)oxy)butan-2-ol and has the structure:



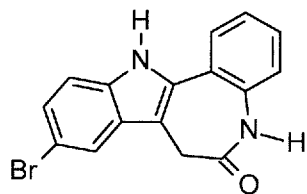
- 15 SNS-032 (BMS-387032, Sunesis) is a selective inhibitor of CDK9, CDK2 and CDK7 (Chen et al., 2009). It is under investigation in clinical trials. In chemical terms, SNS-032 is named N-(5-(((5-(1,1-dimethylethyl)-2-oxazolyl)methyl)thio)-2-thiazolyl)-4-piperidinecarboxamide and has the structure:
- 20



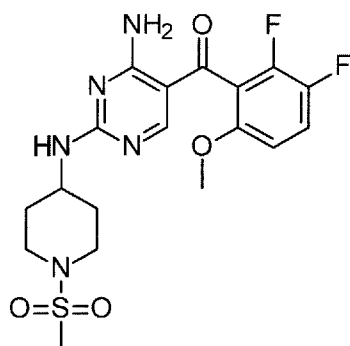
AT7519 (Astex Pharmaceuticals & Novartis) is a broad-range CDK inhibitor, with activity against CDK1, CDK2, CDK4, CDK5, CDK6 and CDK9 as well as glycogen synthase kinase 3 beta (GSK3 β) (Squires et al., 2009). Its therapeutic use is under investigation in clinical trials. In chemical terms, it is named N-(4-Piperidinyl)-4-(2,6-dichlorobenzoylamino)-1H-pyrazole-3-carboxamide hydrochloride and has the structure:



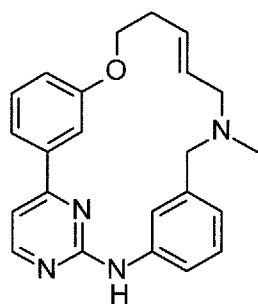
Kenpaullone (NSC 664704) is an ATP-competitive CDK inhibitor which inhibits CDK1, CDK2 and CDK5 (Zaharevitz et al., 1999). In chemical terms, it is named 9-bromo-7,12-dihydrobenzo[2,3]azepino[4,5-b]indol-6(5H)-one and has the structure:



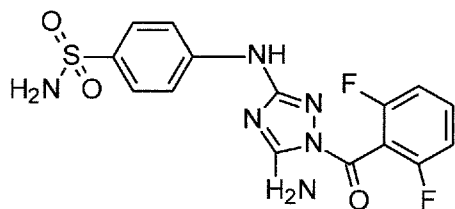
R547 (Ro-4584820, Roche) is an inhibitor selective for CDK1, CDK2 and CDK4 being evaluated in clinical trials (DePinto et al., 2006). In chemical terms, it is named (4-amino-2-((1-(methylsulfonyl)piperidin-4-yl)amino)pyrimidin-5-yl)(2,3-difluoro-6-methoxyphenyl)methanone and has the structure:



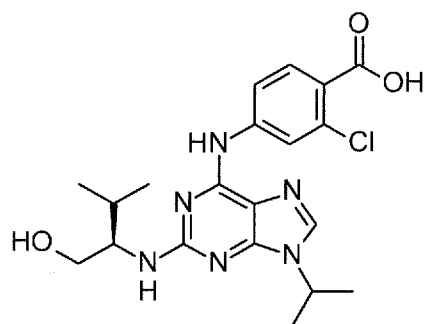
TG02 (SB1317, Tragara Pharmaceuticals) is a pyrimidine-based multi-kinase inhibitor that inhibits CDK1, CDK2, CDK7 and CDK9 that is currently being evaluated clinically (Goh et al., 2012). In chemical terms, it is named
 5 14-methyl-20-oxa-5,7,14,26-tetraazatetracyclo (19.3.1.1(2,6).1(8,12)
)heptacosa-1(25),2(26),3,5,8(27),9,11,16,21,23-decaene and has the structure:



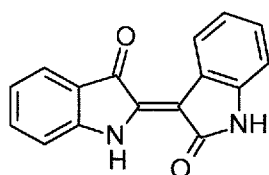
JNJ-7706621 (Johnson and Johnson) is a pan-CDK inhibitor with highest
 10 activity on CDK1 and CDK2 and additional activity on Aurora kinases
 (Emanuel et al., 2005). In chemical terms, it is named 4-((5-amino-1-(2,6-
 difluorobenzoyl)-1H-1,2,4-triazol-3-yl)amino)benzenesulfonamide and has the structure:



15 Purvalanol B is a CDK inhibitor with high selectivity for CDK1, CDK2 and
 CDK5 (Gray et al., 1998). In chemical terms, Purvalanol B is named 2-
 chloro-4-[(2-((2R)-1-hydroxy-3-methylbutan-2-yl)amino)-9-(propan-2-yl)-9H-
 purin-6-yl)amino]benzoic acid and has the structure:

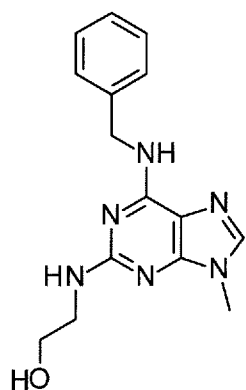


Indirubin and its analogues are known to be potent inhibitors of CDKs (Hoessel et al., 1999). In chemical terms, indirubin is named (3E)-3-(3-oxo-1H-indol-2-ylidene)-1H-indol-2-one and has the structure:

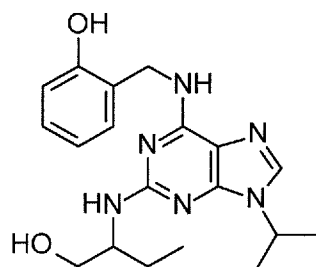


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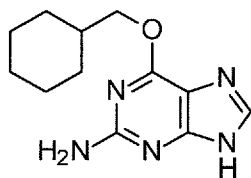
Olomoucine is a purine derivative that competitively inhibits CDK1, CDK2 and CDK5 (Veselý et al., 1994). Chemically it is named 2-[[[9-methyl-6-[(phenylmethyl)amino]-9H-purin-2-yl]amino]-ethanol and has the structure:



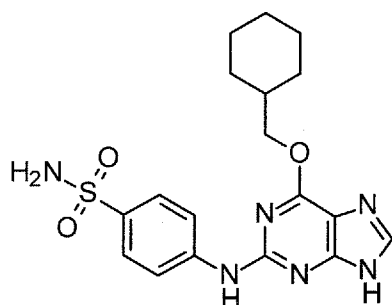
- 10 Olomoucine II is an ATP-competitor that binds and inhibits CDK2, CDK7 and CDK9 activity (Krystof et al., 2005). Chemically, Olomoucine II is named 2-[[[2-[[[(2R)-1-hydroxybutan-2-yl]amino]-9-propan-2-yl]purin-6-yl]amino]methyl]phenol and has the structure:



NU2058 is a guanine based compound that is a competitive inhibitor of CDK1 and CDK2 (Arris et al., 2000). Chemically, NU2058 is named 6-(cyclohexylmethoxy)-9H-purin-2-amine and has the structure:

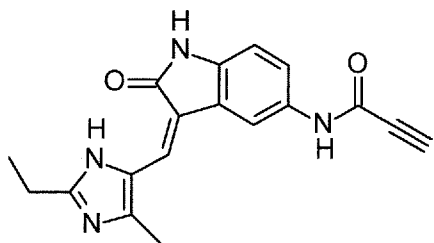


- 5 NU6102 is a selective inhibitor of both CDK1 and CDK2 (Davies et al., 2002). In chemical terms, NU6102 is named 4-[[6-(cyclohexylmethoxy)-7H-purin-2-yl]amino]benzenesulfonamide and has the structure:

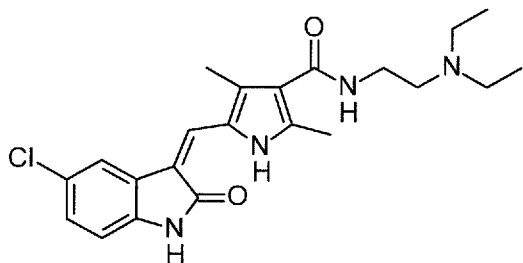


Exemplary NEK inhibitors

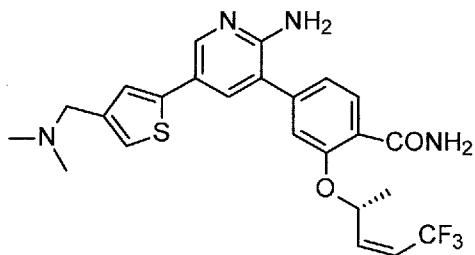
- 10 JH295 is an irreversible, cysteine-targeted inhibitor of NEK2 (Henise & Taunton, 2011). JH295 does not inhibit other mitotic kinases such as CDK1. In chemical terms, JH295 is named (Z)-N-(3-((2-ethyl-4-methyl-1H-imidazol-5-yl)methylene)-2-oxoindolin-5-yl)propiolamide and has the structure:



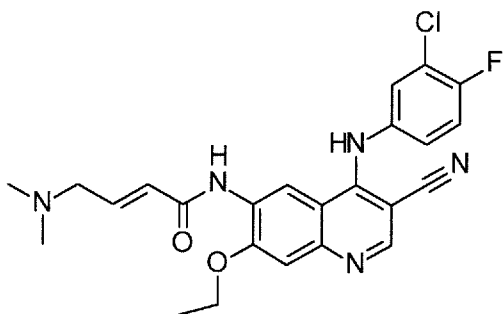
- 15 SU11652 is a pyrrole-indolinone inhibitor that acts as an ATP-competitive tyrosine kinase inhibitor with activity against NEK2 (Rellos et al., 2007). In chemical terms, it is named 5-[(Z)-(5-chloro-2-oxo-1H-indol-3-ylidene)methyl]-N-[2-(diethylamino)ethyl]-2,4-dimethyl-1H-pyrrole-3-carboxamide and has the structure:



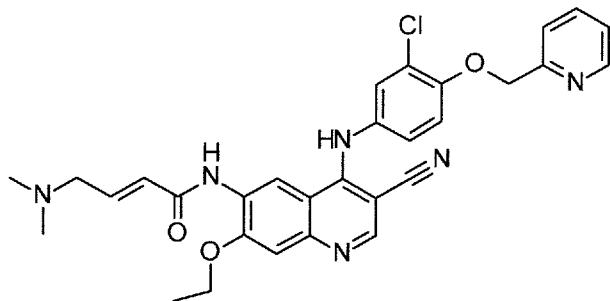
(R)-21 is an aminopyridine that acts as selective and reversible NEK2 inhibitor by targeting the inactive kinase conformation (Innocenti et al., 2012). It was developed by combining key components of two previously discovered classes of NEK2 inhibitors: the aminopyrazines (Whelligan et al., 2010), and benzimidazole compounds (Solanki et al., 2011). In chemical terms, (R)-21 is named (R)-(Z)-4-(2-Amino-5-(4-((dimethylamino)methyl)thiophen-2-yl)pyridin-3-yl)-2-((5,5,5-trifluoropent-3-en-2-yl)oxy)benzamide and has the structure:



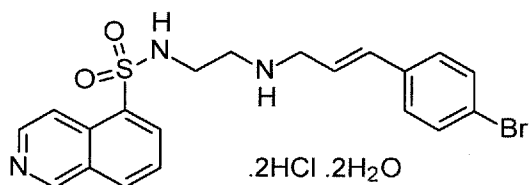
Pelitinib (EKB-569) and neratinib (HKI-272) are epidermal growth factor receptor (EGFR) inhibitors that have recently been found to also inhibit NEK2 (Das et al., 2013). In chemical terms, pelitinib is named (2E)-N-{4-[(3-Chloro-4-fluorophenyl)amino]-3-cyano-7-ethoxy-6-quinolinyl}-4-(dimethylamino)-2-butenamide and has the structure:



In chemical terms, neratinib is named (2E)-N-[4-[[3-chloro-4-[(pyridin-2-yl)methoxy]phenyl]amino]-3-cyano-7-ethoxyquinolin-6-yl]-4-(dimethylamino)but-2-enamide and has the structure:



H-89 is a protein kinase A inhibitor that also inhibits several other kinases, including NEK. H-89 blocks PKA actions through competitive inhibition of the adenosine triphosphate (ATP) site on the PKA catalytic subunit. In chemical terms, H-89 is named N-[2-[[3-(4-Bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide and has the structure:



Pharmaceutical Formulations

Pharmaceutical formulations of a targeted therapeutic agent as described herein are prepared by mixing such targeted therapeutic agent having the desired degree of purity with one or more optional pharmaceutically acceptable carriers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Pharmaceutically acceptable carriers are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol;

salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG). Exemplary pharmaceutically acceptable carriers herein further include

5 hyaluronidase glycoproteins (SHASEGP), for example, human soluble PH-20 hyaluronidase glycoproteins, such as rHuPH20 (HYLENEX®, Baxter International, Inc.). Certain exemplary SHASEGPs and methods of use, including rHuPH20, are described in US Patent Publication Nos. 2005/0260186 and 2006/0104968. In one aspect, a SHASEGP is combined with one or more
10 additional glycosaminoglycanases such as chondroitinases.

Exemplary lyophilized antibody formulations are described in US Patent No. 6,267,958. Aqueous antibody formulations include those described in US Patent No. 6,171,586 and WO2006/044908, the latter formulations including a histidine-acetate buffer.

15 The formulation herein may also contain more than one active ingredients as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such active ingredients are suitably present in combination in amounts that are
20 effective for the purpose intended.

Active ingredients may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-

25 (methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

30 Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the targeted therapeutic agent, which matrices are in the form of shaped articles, e.g. films, or microcapsules.

35 The formulations to be used for *in vivo* administration are generally sterile. Sterility may be readily accomplished, e.g., by filtration through sterile filtration membranes.

40 The foregoing disclosure also applies mutatis mutandis to the formulation of agents that inhibit phosphorylation of the S595 residue of eEF2.

Therapeutic Methods and Compositions

The targeted therapeutic agents and agents that inhibits phosphorylation of the S595 residue of eEF2 provided herein may be used in therapeutic methods.

A targeted therapeutic agent and (where applicable) an agent that inhibits phosphorylation of the S595 residue of eEF2 can be administered by any suitable means, including parenteral, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. Dosing can be by any suitable route, e.g. by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic. Various dosing schedules including but not limited to single or multiple administrations over various time-points, bolus administration, and pulse infusion are contemplated herein.

Targeted therapeutic agents and (where applicable) agents that inhibit phosphorylation of the S595 residue of eEF2 would be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The targeted therapeutic agent and (where applicable) agent that inhibits phosphorylation of the S595 residue of eEF2 need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of targeted therapeutic agent and (where applicable) agent that inhibits phosphorylation of the S595 residue of eEF2 present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as described herein, or about from 1 to 99% of the dosages described herein, or in any dosage and by any route that is empirically/clinically determined to be appropriate.

For the prevention or treatment of disease, the appropriate dosage of a targeted therapeutic agent and (where applicable) agent that inhibits

phosphorylation of the S595 residue of eEF2 of the invention will depend on the type of disease to be treated, the type of targeted therapeutic agent and (where applicable) agent that inhibits phosphorylation of the S595 residue of eEF2, the severity and course of the disease, whether the
5 targeted therapeutic agent and (where applicable) agent that inhibits phosphorylation of the S595 residue of eEF2 is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the targeted therapeutic agent and (where applicable) agent that inhibits phosphorylation of the S595 residue of eEF2, and the
10 discretion of the attending physician. The targeted therapeutic agent and (where applicable) agent that inhibits phosphorylation of the S595 residue of eEF2 are suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 µg/kg to 15 mg/kg (e.g. 0.1mg/kg-10mg/kg) of the targeted
15 therapeutic agent can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the
20 condition, the treatment would generally be sustained until a desired suppression of disease symptoms occurs. One exemplary dosage of the targeted therapeutic agent would be in the range from about 0.05 mg/kg to about 10 mg/kg. Thus, one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 4.0 mg/kg or 10 mg/kg may be administered to the patient. Such doses may be
25 administered intermittently, e.g. every week or every three weeks (e.g. such that the patient receives from about two to about twenty, or e.g. about six doses). An initial higher loading dose, followed by one or more lower doses may be administered. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional
30 techniques and assays.

Articles of Manufacture

In another aspect of the invention, an article of manufacture containing
35 materials useful for the treatment and/or prevention of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, IV solution bags, etc. The containers may be formed from
40 a variety of materials such as glass or plastic. The container holds a composition which is by itself or combined with another composition

effective for treating and/or preventing the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is a targeted therapeutic agent of the invention. The label or package insert indicates that the composition is used for treating the condition of choice. Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises a targeted therapeutic agent of the invention; and (b) a second container with a composition contained therein, wherein the composition comprises an agent that inhibits phosphorylation of the S595 residue of eEF2. The article of manufacture in this embodiment of the invention may further comprise a package insert indicating that the compositions can be used to treat a particular condition. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

Additional statements of the invention

The following numbered paragraphs define various aspects and embodiments of the invention. Except where the context requires otherwise, the disclosure of these numbered paragraphs is intended to be fully combinable with the foregoing disclosure. In particular, preferred features described above are preferred features of the following definitions also.

1. A method for assessing resistance or non-resistance of diseased cells in a patient to treatment with a NAD(+)-diphthamide ADP-ribosyltransferase, the method comprising assaying for serine phosphorylation of eEF2 protein in a sample containing diseased cells, wherein serine phosphorylation of the eEF2 protein is indicative that the diseased cells are resistant to treatment with a NAD(+)-diphthamide ADP-ribosyltransferase.

2. The method of 1, which further includes a step of selecting the patient for treatment with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to the diseased cells of the patient if the diseased cells of the

sample are assessed to be non-resistant to NAD(+)-diphthamide ADP ribosyltransferase.

3. The method of 1 or 2, which further includes a step of deselecting the patient for treatment with a NAD(+)-diphthamide ADP ribosyltransferase if the diseased cells are assessed to be resistant to NAD(+)-diphthamide ADP ribosyltransferase.

4. The method of 2 or 3, which further includes a step, following selection of the patient for treatment, of treating the patient with the targeted therapeutic agent.

5. The method of 1 or 2, which further includes a step of selecting the patient for treatment with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to the diseased cells of the patient in combination with an agent that inhibits phosphorylation of the S595 residue of eEF2 if the diseased cells of the sample are assessed to be resistant to NAD(+)-diphthamide ADP ribosyltransferase.

6. The method of 5, which further includes a step, following the selection of the patient for treatment, of treating the patient with the targeted therapeutic agent in combination with an agent that inhibits phosphorylation of the S595 residue of eEF2.

7. A method for selecting and/or deselecting a patient for treatment with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to diseased cells of the patient, the method comprising:

- (i) assaying for serine phosphorylation of eEF2 protein in a sample containing the diseased cells from the patient; and
- (ii) (a) selecting the patient for treatment with the targeted therapeutic agent if the assay is negative for serine phosphorylation of the eEF2 protein; and/or either
- (ii) (b) (1) deselecting the patient for treatment with the targeted therapeutic agent if the assay is positive for serine phosphorylation of the eEF2 protein; or
- (ii) (b) (2) selecting the patient for treatment with the targeted therapeutic agent in combination with an agent that inhibits phosphorylation of the S595 residue of eEF2 if the assay is positive for serine phosphorylation of the eEF2 protein.

8. The method of 7, further comprising a step, following selection in (ii)(a) of the patient for treatment, of treating the patient with the targeted therapeutic agent.

5

9. The method of 7, further comprising a step, following selection in (ii)(b)(2) of the patient for treatment, of treating the patient with the targeted therapeutic agent in combination with an agent that inhibits phosphorylation of the S595 residue of eEF2.

10

10. A method for treating a patient having a condition that is treatable by cytotoxic activity targeted to diseased cells of the patient, the method comprising:

assaying a sample containing diseased cells from a patient for serine phosphorylation of eEF2 protein; and

15

treating a patient in whose sample the assay is negative for serine phosphorylation of eEF2 protein with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to the diseased cells of the patient.

20

11. A method for treating a patient having a condition that is treatable by cytotoxic activity targeted to diseased cells of the patient, the method comprising:

assaying a sample containing the diseased cells from a patient for serine phosphorylation of eEF2 protein; and

25

treating a patient in whose sample the assay is positive for serine phosphorylation of eEF2 protein with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to the diseased cells of the patient in combination with an agent that inhibits phosphorylation of the S595 residue of eEF2.

30

12. A method for treating a patient having a condition that is treatable by cytotoxic activity targeted to diseased cells of the patient, the method comprising:

assaying for serine phosphorylation of eEF2 protein in a sample containing the diseased cells from the patient;

35

assessing resistance or non-resistance of the diseased cells to treatment with a NAD(+)-diphthamide ADP-ribosyltransferase, wherein serine phosphorylation of the eEF2 protein is indicative that the diseased cells are resistant to treatment with a NAD(+)-diphthamide ADP-ribosyltransferase; and

40

treating a patient whose diseased cells are assessed to be non-resistant with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to the diseased cells of the patient.

5

13. A method for treating a patient having a condition that is treatable by cytotoxic activity targeted to diseased cells of the patient, the method comprising:

10 assaying for serine phosphorylation of eEF2 protein in a sample containing diseased cells from the patient;

15 assessing resistance or non-resistance of the diseased cells to treatment with a NAD(+)-diphthamide ADP-ribosyltransferase, wherein serine phosphorylation of the eEF2 protein is indicative that the diseased cells are resistant to treatment with a NAD(+)-diphthamide ADP-
15 ribosyltransferase; and

20 treating a patient whose diseased cells are assessed to be resistant with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to the diseased cells of the patient in combination with an agent that inhibits
20 phosphorylation of the S595 residue of eEF2.

14. A method for treating a patient having a condition that is treatable by cytotoxic activity targeted to diseased cells of the patient, the method comprising:

25 treating the patient with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to the diseased cells of the patient, wherein the patient is selected for treatment on the basis of a negative assay result for serine phosphorylation of eEF2 protein in a sample containing diseased cells from
30 the patient.

15. A method for treating a patient having a condition that is treatable by cytotoxic activity targeted to diseased cells of the patient, the method comprising:

35 treating the patient with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to the diseased cells of the patient in combination with an agent that inhibits phosphorylation of the S595 residue of eEF2, wherein the patient is selected for treatment on the basis of a positive assay result
40 for serine phosphorylation of eEF2 protein in a sample containing diseased cells from the patient.

16. The method of any one of 1-15, wherein the assay for serine phosphorylation of eEF2 protein is performed with an antibody that selectively binds to eEF2 or a fragment thereof that is phosphorylated at the S595 residue.
17. The method of 16, wherein the binding affinity of the antibody for eEF2 or a fragment thereof that is phosphorylated at the S595 residue is at least 10-fold higher than the binding affinity for eEF2 or a fragment thereof that is non-phosphorylated at the S595 residue.
18. The method of 17, wherein the antibody substantially does not bind to eEF2 or a fragment thereof that is non-phosphorylated at the S595 residue.
19. The method of any one of 16-18, wherein the antibody binds to eEF2 or a fragment thereof that is phosphorylated at the S595 residue with a K_D of 100 nM or less.
20. The method of any one of 16-19, wherein the antibody is a monoclonal antibody having the heavy chain variable domain sequence of SEQ ID NO:53, or a heavy chain variable domain sequence having at least 80%, 85%, 90%, 95%, 98% or 99% identity to SEQ ID NO:53.
21. The method of any one of 16-20, wherein the antibody is a monoclonal antibody having the light chain variable domain sequence of SEQ ID NO:54, or a light chain variable domain sequence having at least 80%, 85%, 90%, 95%, 98% or 99% identity to SEQ ID NO:54.
22. The method of any one of 16-21, wherein the antibody is a monoclonal antibody having the CDR-H1 sequence of SEQ ID NO:55, 56 or 57, or said CDR-H1 sequence with one or more amino acid insertions, deletions and/or substitutions.
23. The method of any one of 16-22, wherein the antibody is a monoclonal antibody having the CDR-H2 sequence of SEQ ID NO:58 or 59, or said CDR-H2 sequence with one or more amino acid insertions, deletions and/or substitutions.
24. The method of any one of 16-23, wherein the antibody is a monoclonal antibody having the CDR-H3 sequence of SEQ ID NO:60, or said CDR-H3

sequence with one or more amino acid insertions, deletions and/or substitutions.

25. The method of any one of 16-24, wherein the antibody is a monoclonal antibody having the CDR-L1 sequence of SEQ ID NO:61, or said CDR-L1 sequence with one or more amino acid insertions, deletions and/or substitutions.
26. The method of any one of 16-25, wherein the antibody is a monoclonal antibody having the CDR-L2 sequence of SEQ ID NO:62, or said CDR-H2 sequence with one or more amino acid insertions, deletions and/or substitutions.
27. The method of any one of 16-26, wherein the antibody is a monoclonal antibody having the CDR-L3 sequence of SEQ ID NO:63, or said CDR-L3 sequence with one or more amino acid insertions, deletions and/or substitutions.
28. The method of any one of 16-27, wherein the antibody is a monoclonal antibody having the heavy chain complementarity-determining region (CDR) sequence H3 of SEQ ID NO:60.
29. The method of 28, wherein the antibody has the heavy chain CDR sequences H2 of SEQ ID NO:58 or 59 and H3 of SEQ ID NO:60.
30. The method of 29, wherein the antibody has the heavy chain CDR sequences H1 of SEQ ID NO:55, 56 or 57, H2 of SEQ ID NO: 58 or 59 and H3 of the SEQ ID NO:60.
31. The method of any one of 16-30, wherein the antibody is a monoclonal antibody having the light chain complementarity-determining region (CDR) sequence L3 of SEQ ID NO:63.
32. The method of 31, wherein the antibody has the light chain CDR sequences L2 and L3 of SEQ ID NOs:62 and 63.
33. The method of 32, wherein the antibody has the light chain CDR sequences L1, L2 and L3 of SEQ ID NOs:61 to 63.
34. The method of any one of 16-33, wherein the antibody is a monoclonal antibody comprising the CDR sequences H1 of SEQ ID NO:55, 56 or 57, H2 of

SEQ ID NO:58 or 59, and H3 of SEQ ID NO:60 and the CDR L1-L3 sequences of the SEQ ID NOs:61 to 63.

- 5 35. The method of any one of 16-34, wherein the antibody is a monoclonal antibody comprising the heavy chain variable domain sequence shown in SEQ ID NO:53 and the light chain variable domain sequence shown in SEQ ID NO:54.
- 10 36. The method of any one of 1-15, wherein the assay for serine phosphorylation of eEF2 protein is performed using a non site-specific anti-phosphoserine antibody.
- 15 37. The method of any one of 16-36, wherein the assay comprises subjecting an extract of the sample to chromatography and contacting one or more chromatography fractions with said antibody.
- 20 38. The method of any one of 16-36, wherein the assay comprises subjecting an extract of the sample to electrophoresis and contacting the electrophoresis gel or a blot thereof with said antibody.
39. The method of any one of 16-36, wherein the assay comprises subjecting an extract of the sample to a sandwich assay comprising said antibody as a capture antibody or detection antibody.
- 25 40. The method of 39, wherein the assay is an ELISA assay.
41. The method of any one of 16-36, wherein the assay comprises subjecting an extract of the sample to a dipstick test comprising said antibody.
- 30 42. The method according to any one of 16-41, wherein said antibody is labelled with a detectable label.
- 35 43. The method of any one of 2-42, wherein the NAD(+)-diphthamide ADP ribosyltransferase of the targeted therapeutic agent is a PE toxin, a DT toxin or a cholix toxin.
- 40 44. The method of 43, wherein the NAD(+)-diphthamide ADP ribosyltransferase of the targeted therapeutic agent is a PE toxin or a DT toxin.

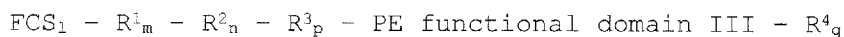
45. The method of 43 or 44, wherein the NAD(+)-diphthamide ADP ribosyltransferase of the targeted therapeutic agent is a PE toxin.

46. The method of any one of 42-45, wherein the PE toxin has a polypeptide sequence comprising a PE functional domain III having at least 50% amino acid sequence identity over the full length of residues 395-601 of SEQ ID NO:1, wherein the PE toxin has cytotoxic activity when introduced into a mammalian cell.

47. The method of 43 or 44, wherein the DT toxin has a polypeptide sequence comprising a DT functional domain I having at least 50% amino acid sequence identity over the full length of residues 1-191 of SEQ ID NO:3, wherein the DT toxin has cytotoxic activity when introduced into a mammalian cell.

48. The method of 43, wherein the cholix toxin has a polypeptide sequence comprising a cholix toxin functional domain III having at least 50% amino acid sequence identity over the full length of residues 424-628 of SEQ ID NO:4, wherein the cholix toxin has cytotoxic activity when introduced into a mammalian cell.

49. The method of any one of 43-46, wherein the PE toxin has the following structure:



wherein:

l, m, n, p and q are each, independently, 0 or 1;

FCS is a furin-cleavable sequence, preferably (i) R-H-R-Q-P-R-G-W-E-Q-L (SEQ ID NO: 6) or a truncated version thereof containing R-Q-P-R (SEQ ID NO: 28), optionally R-Q-P-R (SEQ ID NO: 28), R-H-R-Q-P-R-G-W (SEQ ID NO: 29), R-H-R-Q-P-R-G-W-E (SEQ ID NO: 30), H-R-Q-P-R-G-W-E-Q (SEQ ID NO: 31), or R-Q-P-R-G-W-E (SEQ ID NO: 32); or (ii) R-H-R-S-K-R-G-W-E-Q-L (SEQ ID NO: 18) or a truncated version thereof containing R-S-K-R (SEQ ID NO: 33), optionally R-S-K-R (SEQ ID NO: 33), R-H-R-S-K-R-G-W (SEQ ID NO: 34), H-R-S-K-R-G-W-E (SEQ ID NO: 35), R-S-K-R-G-W-E-Q-L (SEQ ID NO: 36), H-R-S-K-R-G-W-E-Q-L (SEQ ID NO: 37), or R-H-R-S-K-R (SEQ ID NO: 38), wherein the glutamic acid residue corresponding to position 282 of the native PE sequence (where present) is optionally replaced by another residue, preferably glycine, serine, alanine or glutamine;

R¹ is a linker sequence of 1 to 10 amino acids, preferably GGS or GGSGGS (SEQ ID NO: 7);

R² is one or more consecutive amino acid residues of residues 285-364 of SEQ ID NO:1, in which any one or more of residues E285, P290, L294, L297, Y298, L299, R302, R313, N314, P319, D324, E327, E331 and Q332, where present, is/are optionally independently replaced by another amino acid, preferably glycine, serine, alanine or glutamine;

R³ is one or more consecutive amino acid residues of residues 365-394 of SEQ ID NO:1;

PE functional domain III comprises residues 395-613 of SEQ ID NO:1 in which:

(a) some or all of residues 602-608 are optionally deleted, and

(b) residues 609-613 are optionally replaced by another ER localisation sequence, preferably KDEL (SEQ ID NO: 9), REDL (SEQ ID NO:10), RDEL (SEQ ID NO:11) or KEDLK (SEQ ID NO:12), and

(c) any one or more of residues D403, D406, R412, E420, R421, L422, L423, A425, R427, L429, E431, R432, Y439, H440, F443, L444, A446, A447, I450, R456, R458, D461, 463-519 (preferably D463, R467, L477, Y481, R490, R494, R505, R513 and/or L516), E522, R538, E548, R551, L552, T554, I555, L556, W558, R563, R576, D581, D589, K590, Q592, L597 and (where present) K606 is/are optionally independently replaced by another amino acid, preferably glycine, serine, alanine or glutamine, or histidine in the case of L477;

R⁴ is one or more (preferably 1 or 2) additional ER localisation sequences, preferably REDLK (SEQ ID NO: 8), KDEL (SEQ ID NO: 9), REDL (SEQ ID NO:10), RDEL (SEQ ID NO:11) or KEDLK (SEQ ID NO:12).

50. The method of 49, wherein l is 1.

51. The method of 49 or 50, wherein m is 1.

52. The method of any one of 49 to 51, wherein n is 0.

53. The method of any one of 49 to 52, wherein p is 0.

54. The method of any one of 49 to 53, wherein q is 0.

55. The method of any one of 49 to 54, wherein the PE functional domain III includes the combination of mutations R427A/F443A/L477H/R494A/R505A/L552E, or the combination of mutations

R427A/R456A/D463A/R467A/R490A/R505A/R538A, or the combination of mutations R427A/F443A/R456A/D463A/R467A/L477H/R490A/R494A/R505A/R538A/L552E.

56. The method of any one of 49 to 55, wherein the PE toxin comprises the amino acid sequence of SEQ ID NO:64 or SEQ ID NO:65.
57. The method of 56, wherein the amino acid sequence of SEQ ID NO:64 or SEQ ID NO:65 is fused to the C-terminal end of the amino acid sequence of SEQ ID NO:66.
58. The method of any one of 2 to 57, wherein the targeted therapeutic agent comprises a NAD(+)-diphthamide ADP ribosyltransferase coupled to the cell-binding agent as a fusion polypeptide.
59. The method of 58, wherein the NAD(+)-diphthamide ADP ribosyltransferase is directly coupled to the cell-binding agent as a fusion polypeptide.
60. The method of any one of 2 to 59, wherein the cell-binding agent is an antibody.
61. The method of 60, wherein the antibody is an antigen-binding antibody fragment.
62. The method of 60 or 61, wherein the antibody is directed against a tumour-associated antigen.
63. The method of any one of 1 to 62, wherein the diseased cells are pre-cancer, cancer or tumour cells, virally-infected cells or autoimmune effector cells.
64. The method of 63, wherein the diseased cells are pre-cancer, cancer or tumour cells.
65. The method of any one of 10 to 64, wherein the condition is a pre-cancer, cancer, tumour, viral infection or autoimmune disease.
66. The method of 65, wherein the condition is a pre-cancer, cancer or tumour.

67. The method of any one of 5 to 9, 13 and 15 to 66, wherein the agent that inhibits phosphorylation of the S595 residue of eEF2 is an inhibitor of CDK2, CDK1 or NEK.

5 68. The method of 67, wherein the inhibitor of CDK2 and/or CDK1 is a pan-CDK inhibitor.

69. The method of any one of 1 to 68, wherein the patient is human.

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101. A NAD(+)-diphthamide ADP ribosyltransferase for use in a method of medical treatment of a patient from whom a sample containing diseased cells has given a negative result in an assay for serine phosphorylation of eEF2
15 protein.

102. A NAD(+)-diphthamide ADP ribosyltransferase for use in a method of medical treatment of a patient from whom a sample containing diseased cells has been assayed for serine phosphorylation of eEF2 protein and assessed as
20 non-resistant to NAD(+)-diphthamide ADP ribosyltransferase treatment.

103. A NAD(+)-diphthamide ADP ribosyltransferase for use in combination with an agent that inhibits phosphorylation of the S595 residue of eEF2 in a method of medical treatment of a patient from whom a sample containing
25 diseased cells has given a positive result in an assay for serine phosphorylation of eEF2 protein.

104. An agent that inhibits phosphorylation of the S595 residue of eEF2, for use in combination with a NAD(+)-diphthamide ADP ribosyltransferase in
30 a method of medical treatment of a patient from whom a sample containing diseased cells has given a positive result in an assay for serine phosphorylation of eEF2 protein.

105. A NAD(+)-diphthamide ADP ribosyltransferase for use in combination with an agent that inhibits phosphorylation of the S595 residue of eEF2 in
35 a method of medical treatment of a patient from whom a sample containing diseased cells has been assayed for serine phosphorylation of eEF2 protein and assessed as resistant to NAD(+)-diphthamide ADP ribosyltransferase treatment.

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106. An agent that inhibits phosphorylation of the S595 residue of eEF2, for use in combination with a NAD(+)-diphthamide ADP ribosyltransferase in a method of medical treatment of a patient having from whom a sample containing diseased cells has been assayed for serine phosphorylation of eEF2 protein and assessed as resistant to NAD(+)-diphthamide ADP ribosyltransferase treatment.

107. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of any one of 101 to 106, wherein the assay for serine phosphorylation of eEF2 protein was performed with an antibody that selectively binds to eEF2 or a fragment thereof that is phosphorylated at the S595 residue.

108. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of 107, wherein the binding affinity of the antibody for eEF2 or a fragment thereof that is phosphorylated at the S595 residue is at least 10-fold higher than the binding affinity for eEF2 or a fragment thereof that is non-phosphorylated at the S595 residue.

109. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of 108, wherein the antibody substantially does not bind to eEF2 or a fragment thereof that is non-phosphorylated at the S595 residue.

110. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of any one of 107-109, wherein the antibody binds to eEF2 or a fragment thereof that is phosphorylated at the S595 residue with a K_D of 100 nM or less.

111. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of any one of 107-110, wherein the antibody is a monoclonal antibody having the heavy chain variable domain sequence of SEQ ID NO:53, or a heavy chain variable domain sequence having at least 80%, 85%, 90%, 95%, 98% or 99% identity to SEQ ID NO:53.

112. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of any one of 107-111, wherein the antibody is a monoclonal antibody having the light chain variable domain sequence of SEQ ID NO:54, or a light chain variable

domain sequence having at least 80%, 85%, 90%, 95%, 98% or 99% identity to SEQ ID NO:54.

- 5 113. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of any one of 107-112, wherein the antibody is a monoclonal antibody having the CDR-H1 sequence of SEQ ID NO:55, 56 or 57, or said CDR-H1 sequence with one or more amino acid insertions, deletions and/or substitutions.
- 10 114. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of any one of 107-113, wherein the antibody is a monoclonal antibody having the CDR-H2 sequence of SEQ ID NO:58 or 59, or said CDR-H2 sequence with one or more amino acid insertions, deletions and/or substitutions.
- 15 115. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of any one of 107-114, wherein the antibody is a monoclonal antibody having the CDR-H3 sequence of SEQ ID NO:60, or said CDR-H3 sequence with one or more amino acid insertions, deletions and/or substitutions.
- 20 116. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of any one of 107-115, wherein the antibody is a monoclonal antibody having the CDR-L1 sequence of SEQ ID NO:61, or said CDR-L1 sequence with one or more amino acid insertions, deletions and/or substitutions.
- 25 117. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of any one of 107-116, wherein the antibody is a monoclonal antibody having the CDR-L2 sequence of SEQ ID NO:62, or said CDR-H2 sequence with one or more amino acid insertions, deletions and/or substitutions.
- 30 118. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of any one of 107-117, wherein the antibody is a monoclonal antibody having the CDR-L3 sequence of SEQ ID NO:63, or said CDR-L3 sequence with one or more amino acid insertions, deletions and/or substitutions.
- 35 119. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of any one of

107-118, wherein the antibody is a monoclonal antibody having the heavy chain complementarity-determining region (CDR) sequence H3 of SEQ ID NO:60.

120. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of 119, wherein the antibody has the heavy chain CDR sequences H2 of SEQ ID NO:58 or 59 and H3 of SEQ ID NO:60.

121. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of 120, wherein the antibody has the heavy chain CDR sequences H1 of SEQ ID NO:55, 56 or 57, H2 of SEQ ID NO: 58 or 59 and H3 of the SEQ ID NO:60.

122. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of any one of 107-121, wherein the antibody is a monoclonal antibody having the light chain complementarity-determining region (CDR) sequence L3 of SEQ ID NO:63.

123. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of 122, wherein the antibody has the light chain CDR sequences L2 and L3 of SEQ ID NOs:62 and 63.

124. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of 123, wherein the antibody has the light chain CDR sequences L1, L2 and L3 of SEQ ID NOs:61 to 63.

125. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of any one of 107-124, wherein the antibody is a monoclonal antibody comprising the CDR sequences H1 of SEQ ID NO:55, 56 or 57, H2 of SEQ ID NO:58 or 59, and H3 of SEQ ID NO:60 and the CDR L1-L3 sequences of the SEQ ID NOs:61 to 63.

126. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of any one of 107-125, wherein the antibody is a monoclonal antibody comprising the heavy chain variable domain sequence shown in SEQ ID NO:53 and the light chain variable domain sequence shown in SEQ ID NO:54.

127. The NAD(+)-diphthamide ADP ribosyltransferase for use or agent that inhibits phosphorylation of the S595 residue of eEF2 for use of any one of 101 to 106, wherein the assay for serine phosphorylation of eEF2 protein was performed using a non site-specific anti-phosphoserine antibody.

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128. A pharmaceutical combination comprising a NAD(+)-diphthamide ADP ribosyltransferase and an agent that inhibits phosphorylation of the S595 residue of eEF2.

10 129. The pharmaceutical combination of 128, which is for use in the treatment of patients whose diseased cells have given a positive result in an assay for serine phosphorylation of eEF2 protein and/or whose diseased cells have been assayed for serine phosphorylation of eEF2 protein and assessed as resistant to NAD(+)-diphthamide ADP ribosyltransferase

15 treatment.

130. A NAD(+)-diphthamide ADP ribosyltransferase for use in a method of medical treatment, wherein the treatment comprises administering the NAD(+)-diphthamide ADP ribosyltransferase in combination with an agent that

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131. An agent that inhibits phosphorylation of the S595 residue of eEF2 for use in a method of medical treatment, wherein the treatment comprises administering the agent that inhibits phosphorylation of the S595 residue

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132. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of any one of 101 to 131, wherein the NAD(+)-

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133. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of 132, wherein the PE toxin has a polypeptide sequence comprising a PE functional domain III having at least 50% amino acid sequence identity over the full length of residues 395-601 of SEQ ID NO:1, wherein the PE toxin has cytotoxic activity when introduced into a

40

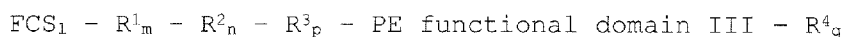
134. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of 132, wherein the DT toxin has a polypeptide sequence comprising a DT functional domain I having at least 50% amino acid sequence identity over the full length of residues 1-191 of SEQ ID NO:3, wherein the DT toxin has cytotoxic activity when introduced into a mammalian cell.

135. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of 132, wherein the cholix toxin has a polypeptide sequence comprising a cholix toxin functional domain III having at least 50% amino acid sequence identity over the full length of residues 424-628 of SEQ ID NO:4, wherein the cholix toxin has cytotoxic activity when introduced into a mammalian cell.

136. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of any one of 132-135, wherein the NAD(+)-diphthamide ADP ribosyltransferase is a PE toxin or a DT toxin.

137. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of 132 or 133, wherein the NAD(+)-diphthamide ADP ribosyltransferase is a PE toxin.

138. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of 137, wherein the PE toxin has the following structure:



wherein:

1, m, n, p and q are each, independently, 0 or 1;

FCS is a furin-cleavable sequence, preferably (i) R-H-R-Q-P-R-G-W-E-Q-L (SEQ ID NO: 6) or a truncated version thereof containing R-Q-P-R (SEQ ID NO: 28), optionally R-Q-P-R (SEQ ID NO: 28), R-H-R-Q-P-R-G-W (SEQ ID NO: 29), R-H-R-Q-P-R-G-W-E (SEQ ID NO: 30), H-R-Q-P-R-G-W-E-Q (SEQ ID NO: 31), or R-Q-P-R-G-W-E (SEQ ID NO: 32); or (ii) R-H-R-S-K-R-G-W-E-Q-L (SEQ ID NO: 18) or a truncated version thereof containing R-S-K-R (SEQ ID NO: 33),

optionally R-S-K-R (SEQ ID NO: 33), R-H-R-S-K-R-G-W (SEQ ID NO: 34), H-R-S-K-R-G-W-E (SEQ ID NO: 35), R-S-K-R-G-W-E-Q-L (SEQ ID NO: 36), H-R-S-K-R-G-W-E-Q-L (SEQ ID NO: 37), or R-H-R-S-K-R (SEQ ID NO: 38), wherein the glutamic acid residue corresponding to position 282 of the native PE sequence (where present) is optionally replaced by another residue, preferably glycine, serine, alanine or glutamine;

R¹ is a linker sequence of 1 to 10 amino acids, preferably GGS or GGSGGS (SEQ ID NO: 7);

R² is one or more consecutive amino acid residues of residues 285-364 of SEQ ID NO:1, in which any one or more of residues E285, P290, L294, L297, Y298, L299, R302, R313, N314, P319, D324, E327, E331 and Q332, where present, is/are optionally independently replaced by another amino acid, preferably glycine, serine, alanine or glutamine;

R³ is one or more consecutive amino acid residues of residues 365-394 of SEQ ID NO:1;

PE functional domain III comprises residues 395-613 of SEQ ID NO:1 in which:

(a) some or all of residues 602-608 are optionally deleted, and

(b) residues 609-613 are optionally replaced by another ER localisation sequence, preferably KDEL (SEQ ID NO: 9), REDL (SEQ ID NO:10), RDEL (SEQ ID NO:11) or KEDLK (SEQ ID NO:12), and

(c) any one or more of residues D403, D406, R412, E420, R421, L422, L423, A425, R427, L429, E431, R432, Y439, H440, F443, L444, A446, A447, I450, R456, R458, D461, 463-519 (preferably D463, R467, L477, Y481, R490, R494, R505, R513 and/or L516), E522, R538, E548, R551, L552, T554, I555, L556, W558, R563, R576, D581, D589, K590, Q592, L597 and (where present) K606 is/are optionally independently replaced by another amino acid, preferably glycine, serine, alanine or glutamine, or histidine in the case of L477;

R⁴ is one or more (preferably 1 or 2) additional ER localisation sequences, preferably REDLK (SEQ ID NO: 8), KDEL (SEQ ID NO: 9), REDL (SEQ ID NO:10), RDEL (SEQ ID NO:11) or KEDLK (SEQ ID NO:12).

139. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of 138, wherein 1 is 1.

140. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of 138 or 139, wherein m is 1.

141. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of any one of 138-140, wherein n is 0.

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142. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of any one of 138-141, wherein p is 0.

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143. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of any one of 138-142, wherein q is 0.

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144. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of any one of 138-143, wherein the PE functional domain III includes the combination of mutations

R427A/F443A/L477H/R494A/R505A/L552E, or the combination of mutations

R427A/R456A/D463A/R467A/R490A/R505A/R538A, or the combination of mutations

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R427A/F443A/R456A/D463A/R467A/L477H/R490A/R494A/R505A/R538A/L552E.

145. The NAD(+)-diphthamide ADP ribosyltransferase for use of any one of 138 to 144, wherein the PE toxin comprises the amino acid sequence of SEQ ID NO:64 or SEQ ID NO:65.

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146. The NAD(+)-diphthamide ADP ribosyltransferase for use of 145, wherein the amino acid sequence of SEQ ID NO:64 or SEQ ID NO:65 is fused to the C-terminal end of the amino acid sequence of SEQ ID NO:66.

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147. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of any one of 101 to 146, wherein the NAD(+)-diphthamide ADP ribosyltransferase is coupled to a cell-binding agent targeted to diseased cells of the patient.

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148. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of any one of 101 to 147, wherein the NAD(+)-diphthamide ADP ribosyltransferase is coupled to the cell-binding agent as

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a fusion polypeptide.

149. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of 148, wherein the NAD(+)-diphthamide ADP ribosyltransferase is directly coupled to the cell-binding agent as a fusion polypeptide.

150. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of any one of 147 to 149, wherein the cell-binding agent is an antibody.

151. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of 150, wherein the antibody is an antigen-binding antibody fragment.

152. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of 150 or 151, wherein the antibody is directed against a tumour-associated antigen.

153. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of any one of 101 to 152, wherein the diseased cells are pre-cancer, cancer or tumour cells, virally-infected cells or autoimmune effector cells.

154. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of 153, wherein the diseased cells are pre-cancer, cancer or tumour cells.

155. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of any one of 101 to 154, which is for use in the treatment of a pre-cancer, cancer, tumour, viral infection or autoimmune disease.

156. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the

pharmaceutical combination of 155, which is for use in the treatment of a pre-cancer, cancer or tumour.

157. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of any one of 103 to 156, wherein the agent that inhibits phosphorylation of the S595 residue of eEF2 is an inhibitor of CDK2, CDK1 or NEK.

158. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of 157, wherein the inhibitor of CDK2 and/or CDK1 is a pan-CDK inhibitor.

159. The NAD(+)-diphthamide ADP ribosyltransferase for use, the agent that inhibits phosphorylation of the S595 residue of eEF2 for use, or the pharmaceutical combination of any one of 101 to 158, which is for use in the treatment of a human patient.

201. A NAD(+)-diphthamide ADP ribosyltransferase for use in a method of medical treatment of a patient having a condition that is treatable by cytotoxic activity targeted to diseased cells of the patient, wherein the method is as defined in any one of 4, 6 and 8 to 69.

202. An agent that inhibits phosphorylation of the S595 residue of eEF2 for use in a method of medical treatment according to any one of 6, 9, 11, 13 and 15 to 69.

301. A monoclonal antibody that selectively binds to eEF2 or a fragment thereof that is phosphorylated at the S595 residue.

302. The monoclonal antibody of 301, having a binding affinity for eEF2 or a fragment thereof that is phosphorylated at the S595 residue at least 10-fold higher than the binding affinity for eEF2 or a fragment thereof that is non-phosphorylated at the S595 residue.

303. The monoclonal antibody of 301 or 302, which substantially does not bind to eEF2 or a fragment thereof that is non-phosphorylated at the S595 residue.

5 304. The monoclonal antibody of any one of 301 to 303, wherein the antibody binds to eEF2 or a fragment thereof that is phosphorylated at the S595 residue with a K_D of 100 nM or less.

10 305. The monoclonal antibody of any one of 301 to 304, having the heavy chain variable domain sequence of SEQ ID NO:53, or a heavy chain variable domain sequence having at least 80%, 85%, 90%, 95%, 98% or 99% identity to SEQ ID NO:53.

15 306. The monoclonal antibody of any one of 301 to 305, having the light chain variable domain sequence of SEQ ID NO:54, or a light chain variable domain sequence having at least 80%, 85%, 90%, 95%, 98% or 99% identity to SEQ ID NO:54.

20 307. The monoclonal antibody of any one of 301 to 306, having the CDR-H1 sequence of SEQ ID NO:55, 56 or 57, or said CDR-H1 sequence with one or more amino acid insertions, deletions and/or substitutions.

25 308. The monoclonal antibody of any one of 301 to 307, having the CDR-H2 sequence of SEQ ID NO:58 or 59, or said CDR-H2 sequence with one or more amino acid insertions, deletions and/or substitutions.

30 309. The monoclonal antibody of any one of 301 to 308, having the CDR-H3 sequence of SEQ ID NO:60, or said CDR-H3 sequence with one or more amino acid insertions, deletions and/or substitutions.

310. The monoclonal antibody of any one of 301 to 309, having the CDR-L1 sequence of SEQ ID NO:61, or said CDR-L1 sequence with one or more amino acid insertions, deletions and/or substitutions.

35 311. The monoclonal antibody of any one of 301 to 310, having the CDR-L2 sequence of SEQ ID NO:62, or said CDR-H2 sequence with one or more amino acid insertions, deletions and/or substitutions.

40 312. The monoclonal antibody of any one of 301 to 311, having the CDR-L3 sequence of SEQ ID NO:63, or said CDR-L3 sequence with one or more amino acid insertions, deletions and/or substitutions.

313. The monoclonal antibody of any one of 301 to 312, having the heavy chain complementarity-determining region (CDR) sequence H3 of SEQ ID NO:60.

5 314. The monoclonal antibody of 313, having the heavy chain CDR sequences H2 of SEQ ID NO:58 or 59 and H3 of SEQ ID NO:60.

10 315. The monoclonal antibody of 314, having the heavy chain CDR sequences H1 of SEQ ID NO:55, 56 or 57, H2 of SEQ ID NO: 58 or 59 and H3 of the SEQ ID NO:60.

316. The monoclonal antibody of any one of 301 to 315, having the light chain complementarity-determining region (CDR) sequence L3 of SEQ ID NO:63.

15 317. The monoclonal antibody of 316, having the light chain CDR sequences L2 and L3 of SEQ ID NOs:62 and 63.

20 318. The monoclonal antibody of 317, having the light chain CDR sequences L1, L2 and L3 of SEQ ID NOs:61 to 63.

319. The monoclonal antibody of any one of 301 to 318, comprising the CDR sequences H1 of SEQ ID NO:55, 56 or 57, H2 of SEQ ID NO:58 or 59, and H3 of SEQ ID NO:60 and the CDR L1-L3 sequences of the SEQ ID NOs:61 to 63.

25 320. The monoclonal antibody of any one of 301 to 319, comprising the heavy chain variable domain sequence shown in SEQ ID NO:53 and the light chain variable domain sequence shown in SEQ ID NO:54.

30 321. The monoclonal antibody according to any one of 301 to 320, which is labelled with a detectable label.

322. The monoclonal antibody according to 321, wherein the detectable label is an enzyme, a fluorescent label, a radiolabel, an electroluminescent label or biotin.

35 323. Use of the monoclonal antibody according to any one of 301 to 322 in an *in vitro* method of assessing resistance or non-resistance of a cell population to NAD(+)-diphthamide ADP-ribosyltransferase treatment.

40 324. Use of the monoclonal antibody according to any one of 301 to 322 in the method of any one of 1 to 69.

The invention is further illustrated in the following examples, which are not intended to limit the invention in any way.

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Example 1: S595 phosphorylation changes the structure of eEF2 in the vicinity of the diphthamide

10 A model for human eEF2 in complex with *Pseudomonas* exotoxin A was created based on the X-ray structure PDB:3B82 (complex of *S. cerevisiae* eEF2, PE and NAD⁺, Jørgensen R et al. 2008b). Human and yeast eEF2 share a sequence identity of 65.7%. Mapping of the sequence homology on this eEF2-PE structure reveals the protein surface that interacts with PE to be identical between human and yeast, including H715 and S595. Human eEF2 is 15 taken from PDB:3J3A, which is a cryo-EM based model of the human 80s ribosome.

20 In this model, human eEF2 has a different conformation than yeast eEF2 in complex with PE. The tertiary structure of the individual domains however is identical. The structure of yeast eEF2 is used as a template for the reorganization of the human eEF2 parts into a "PE-binding competent" form. This is based on the assumption that the interaction mode between eEF2 and PE is mechanistically conserved. None of the amino acid changes that we implemented to humanize the yeast-derived structure generated structural 25 incompatibilities, or generated alterations in proximity to the phosphorylation sites at T56 and S595, or alterations close to H715 and its attached diphthamide. The diphthamide modification was modeled by hand.

30 The phosphorylation site T56 is located on a loop that is not visible in the yeast eEF2:PE structure and thus disordered. Consequently, the loop A49-R66 was modeled and minimized by molecule typing with CHARMM forcefield and minimizing with a conjugate gradient method including 800 steps, an RMS gradient of 0.1 and an energy change value of 0.0 (note: we observed a discrepancy in position designation between sequence files and literature, 35 with T56 actually being in sequence position 57 in the sequence. For unambiguous identification, we call 'T56' the amino acid that is underlined in the sequence stretch AGETRFTDTR (SEQ ID NO: 5) of eEF2).

The model of human eEF2 (**Figure 1a**) reveals the eEF2K phosphorylation site T56 to be distant from S595 (>55Å) and His715 and its attached diphthamide 40 (>60Å). However, S595 is in close proximity to H715 (10.4 Å with attached

diphthamide (**Figure 1a**). The corresponding model of human eEF2 complexed with PE (**Figure 1b**) shows that both S595 and H715 diphthamide are located in the eEF2-PE interface, i.e. in the region of complex formation between eEF2 and toxin. Hizli et al., 2013 have shown that S595 becomes phosphorylated by CDK-2 and thereby modulates eEF2K mediated T56 phosphorylation. Surprisingly, we found that phosphoserine is not tolerable at the position and inward-facing orientation of S595 in the eEF2 structure: Phospho-S595 placed into the structure results in multiple orbital overlaps of the phosphate group, e.g. with N597 and N600 of eEF2 (**Figure 1c**). The same incompatibilities of pS595 are observed with the original yeast eEF2-PE complex 3B82 or the human eEF2 structure 3J3A. S595 is part of a short loop which contains a proline (P596). This loop may undergo structural alterations that change the orientation of the serine and thereby may permit its phosphorylation. For example, a structurally compatible trans-cis isomerization of P596 would 'flip' the orientation of S595, making it accessible on the surface and permit phosphorylation (**Figure 1d**). However, local structural alterations as consequence of S595 phosphorylation also disturb the eEF2-PE interface, i.e. in the region of complex formation between eEF2 and ADP-ribosylating toxins (**Figure 1d**).

Example 2: ADP-ribosylation modulates phosphorylation at S595

To analyze if ADP ribosylation influences phosphorylation at S595 or T56, cell extracts containing eEF2 protein was first ADP-ribosylated with PE using Bio-NAD as substrate and subsequently analyzed by anti-phosphoserine immunoprecipitation and eEF2 Western blot analyses. Thereby, presence of total eEF2 in these extracts as well as presence of serine-phosphorylated eEF2 could be assessed.

Figure 2 shows that eEF2 signals that can be detected with anti-pS antibodies are diminished when cells have been exposed to toxin and eEF2 is ADP-ribosylated. The amount of total eEF2 appears to be the same in these extracts. This indicates that ADP-ribosylation at H715 diphthamide interferes with serine-phosphorylation of eEF2, in particular at the close-by position S595. A control experiment in which cells that do not contain diphthamide (dphlko cells) were exposed to toxin revealed no influence of toxin treatment on serine phosphorylation of eEF2 (**Figure 2**). Thus, serine-phosphorylation of eEF2 is modulated specifically by the action of the toxin on diphthamide modified eEF2.

Example 3: MCF-7 cells with homozygous knockouts of the diphthamide synthesis gene DPH1 are resistant to PE and DT

5 The human DPH1 gene, also known as OVCA1, is located on chromosome 17 and encodes an enzyme of the biosynthesis pathway for the H715 diphthamide modification of eEF2. Diphthamide biosynthesis consists of three steps. In the first step, four enzymes (Dph1-Dph4) are necessary to transfer a 3-amino-3-carboxypropyl (ACP) group to H715. Next, the methyltransferase Dph5
10 is required to generate diphthine, which is then converted to diphthamide by Dph6 and Dph7 catalyzed amidation (Liu et al. 2004).

To generate cells lacking DPH1 functionality, gene knockouts were generated in MCF-7. The technology used bases on zinc finger nucleases (ZFN,
15 Flisikowska et al. 2011). ZFN specific for DPH1 were transfected in MCF-7, grown for three days to express the nucleases and thereby induce mutations.

Diphthamide modified eEF2 is the target of PE and DT. Within cells, the toxins catalyze ADP-ribosylation of the diphthamide of eEF2 using NAD as
20 ADP-ribosyl donor. The consequence of ADP-ribosylation of eEF2 is an arrest of protein translation and cell death. If Dph1 is essential for diphthamide synthesis and functionally unique (i.e. loss of functionality cannot be taken over by other human genes), homozygous knockouts should fail to generate diphthamide and thus be resistant to PE and DT. In
25 consequence, toxin selection of transfected cells may be applicable to (i) analyze if DPH1 knockout generates toxin resistances in MCF-7 and (ii) enrich for or select MCF-7 cells and clones that carry DPH1 knockouts. The procedure of ZFN-transfection and subsequent toxin selection is summarized in **Figure 3A**. Despite the extensive complexity of the novel
30 procedure (multi-step approach over an extended time period), MCF-7 cells that carry mutations of the DPH1 gene could be obtained. The ZFN-induced DPH1 alterations generated homozygous knockouts, as confirmed by sequencing of the mutated alleles of the DPH1 knockouts (two out of frame mutations in two alleles with no functional wildtype allele remaining, **Figure 3B**).

35 A comparison of the sensitivity of DPH1 knockout cells with parental MCF7 cells indicated that knockout cells have lost sensitivity to the ADP ribosylating toxins PE and DT (**Figure 3C**). Parental MCF-7 cells are quite sensitive to the toxins, whereas the knockout cells are resistant to PE and
40 DT even at highest toxin concentrations. Moreover, resistance is specific for and restricted to eEF2 ADP-ribosylating toxins because other protein

synthesis inhibitors such as cycloheximide (CHX) are still active in the knockout cells to the same degree as in the parental cell line.

This indicates that the functionality of the DPH1 gene product is essential for diphthamide modification on eEF2. In consequence, cells with homozygous loss of function of DPH1 (as in the generated MCF-7-Dph1ko) do not carry a diphthamide on H715 of eEF2.

Example 4: Diphthamide is required for ADP-ribosylation but not for serine or T56 phosphorylation of eEF2

MCF-7 and dph1-ko MCF-7 cells and cell extracts were used to analyze if the diphthamide modification at H715 influences not only ADP-ribosylation but also phosphorylation at S595 or T56 of eEF2.

To analyze ADP-ribosylation at H715, extracts containing eEF2 with (from MCF-7) or without diphthamide (from MCF7dph1ko) were treated with PE or DT using biotinylated NAD as substrate. ADP-ribosylation of eEF2 was detected by enzyme-conjugated streptavidin which binds biotinylated ADP-ribose attached to the diphthamide. **Figure 4A** demonstrates that eEF2 of parent MCF-7 cells becomes ADP-ribosylated upon exposure to ADP-ribosylating toxin PE. In contrast, eEF2 of MCF7dph1ko cells is not ADP-ribosylated by PE. Similar results were obtained with DT (data not shown). This confirms that eEF2 of homozygous DPH1 knockout cells does not carry a functional diphthamide, and therefore is not modified by ADP-ribosylating toxins.

To analyze if H715 diphthamide is necessary for eEF2 phosphorylation, serine-phosphorylated eEF2 was immunoprecipitated from extracts of MCF-7 and MCF7dph1ko cells by anti-pSer antibodies and subsequently subjected to Western blot analyses to determine its eEF2 serine phosphorylation status. **Figure 4B** demonstrates that eEF2 of Dph1 ko cells (i.e. without diphthamide) can be immunoprecipitated to the same degree with anti-pS antibodies. This indicates that the diphthamide is not necessary for serine phosphorylation of eEF2.

To analyze T56 phosphorylation, T56-phosphorylated eEF2 was detected in extracts of MCF-7 and MCF7dph1ko cells by Western Blot analyses with anti-pT56 antibodies. The eEF2 and pT56-eEF2 status was analyzed in untreated cells, as well as in cells that were incubated with NH125 (1-hexadecyl-2-methyl-3-(phenylmethyl)-1H-imidazolium iodide; CAS No: 278603-08-0). NH125

stimulates T56 phosphorylation (Chen Z et al. 2011). **Figure 4C** shows that extracts of both cell lines (irrespective of eEF2 diphthamidylation) contain T56 phosphorylated eEF2. EEF2 of cells treated with NH125 exhibited stronger pT56 signals compared to eEF2 of untreated cells, regardless of presence or absence of diphthamide. These results indicate that the diphthamide on eEF2 is not necessary for T56 phosphorylation, and that the diphthamide on eEF2 is not necessary for NH125-mediated stimulation of T56 phosphorylation.

Example 5: ADP-ribosylation of eEF2 induces genes that are part of the amino acid depletion response

S595 phosphorylation modulates EEF2K-mediated T56 phosphorylation (Hizli et al. 2013). T56 phosphorylation in turn causes protein synthesis inhibition as well as induction of pro-survival pathways. In particular, the amino acid deprivation response is triggered by EEF2K-mediated T56 phosphorylation (Leprivier et al. 2013). Gene expression patterns of this stress response are known and appear to be generally similar in different cells (Shan J et al. 2010; Balasubramanian et al. 2013; Kilberg MS et al. 2012).

The response of MCF-7 cells upon 7hr exposure to the catalytic domain of PE at IC50 concentration (5uM) was analyzed by genome-wide transcriptional profiling (Affimetrix microarray analyses). For that, a truncated PE derivative (PE38) without cell binding domain which enters cells nonspecifically at high concentration was applied. This eliminates potential signaling effects of targeted cell binding (mediated by the deleted domain I of full lengths PE), thereby focusing on consequences of its ADP-ribosylating activity. A toxin concentration at IC50 (5uM for PE38) and exposure for 7 hrs were chosen to allow some time for toxin uptake and intracellular activity, yet be sufficiently early to have the cells still adherent and be able to prepare mRNA of sufficient quality. As controls, cells were exposed (for 7hrs at IC50) to toxins that kill by different mechanisms: cycloheximide (inhibits tRNA translocation and protein synthesis), alpha amanitin (inhibits transcription) and geldanamycin (inhibits protein folding). Principal component analyses (**Figure 5a**) indicated that cells respond specifically to different toxins, i.e. they elicit different transcriptional responses corresponding to their different mode of action. The response of the protein synthesis inhibitor

PE and cycloheximide was more related to each other, while that of amanitin and geldanamycin were divergent from those as well as from each other.

The PE-associated mRNA profile indicated that ADP-ribosylation of eEF2 and protein synthesis inhibition lead to the induction of EGR1, ATF3, JUN, FOS, and associated genes which are directly linked to each other by known pathway connections (Ingenuity pathway analyses, **Figure 6b**). This stress pathway' and the genes associated with it are also major components of transcription profiles that were previously described for the amino acid deprivation response (Shan J et al. 2010; Balasubramanian et al. 2013; Kilberg MS et al. 2012).

Example 6: ADP-ribosylation of eEF2 is more stable than S595 and T56 phosphorylation

EEF2K-mediated phosphorylation of eEF2 at T56 as well as ADP-ribosylation of eEF2 at diphthamide both stall protein synthesis. Also, both modifications are associated with similar transcriptional responses (as described in Example 5). However, eEF2 phosphorylation has been described as event that triggers pro-survival pathways in cells (Leprivier et al. 2013), while ADP-ribosylation causes cell death. One explanation for this paradox could be that a phosphorylation-mediated arrest of protein synthesis is reversible while ADP-ribosylation mediated arrest may be irreversible or long lasting.

To compare the stability of the two different types of eEF2 modification, recombinant eEF2 which was phosphorylated by CDK-2 at S595, or by EEF2K at T56, and then incubated in MCF-7 cell extracts without addition of any inhibitor at 37°C. The degree of eEF2 phosphorylation at S595 or T56 was subsequently determined after different time of incubation by eEF2 immunoprecipitation and pS- or pT56-specific Western blots as described above. In a similar manner, recombinant eEF2 was ADP-ribosylated by PE with Bio-NAD as substrate, then incubated in MCF-7 cell extracts with addition of excess NAD (to quench any remaining activity of carryover PE in the extract) without addition of any other inhibitor at 37°C. The degree of ADP-ribosylation was determined after different time of incubation by eEF2 immunoprecipitation followed by detection with enzyme-labeled streptavidin as described above.

The results of these analyses demonstrate that eEF2 phosphorylation is quite labile, with signal decreases (dephosphorylation) detectable in less than 15 minutes, and complete loss of pT detection after 30 min (**Figure 6a**). It is known to experts in the field that serine phosphorylation is as
5 instable as threonine phosphorylation. Therefore, disappearance of the phospho-Thr56 signals also reflect the instability of the serine-phosphorylation. In contrast to the phosphorylation-instability, ADP-ribosylation of eEF2 appears to be rather stable: ADP-ribosylated eEF2 was detectable with no or only minimal signal decrease for several hours
10 (**Figure 6b**).

Thus, one relevant difference between phosphorylation and ADP-ribosylation of eEF2, and subsequent synthesis arrest and stress pathway induction is the stability of these modifications. Instable phosphorylation may provide
15 a transient pro-survival 'emergency break' of translation under stress conditions, while ADP-ribosylation irreversibly blocks protein synthesis and leads to cell death.

20 Example 7: Alteration of S595 interferes with toxin-mediated ADP-ribosylation of eEF2

It is shown in Example 2 that ADP-ribosylation of H715 diphthamide interferes with phosphorylation of the close by positioned S595. Also,
25 S595 phosphorylation causes structural alterations in the H715-diphthamide containing region that provides the interface for ADP-ribosylating toxins (Example 1). Hence, S595 phosphorylation alters the toxin-binding interface and thereby should modulate toxin access to eEF2. A direct experimental proof that S595 phosphorylation modulates toxin mediated ADP-
30 ribosylation is hampered by technical limitations as phosphorylation of S595 of eEF2 is instable and becomes rapidly lost in cell extracts by dephosphorylation (Example 6).

To overcome this limitation, eEF2 derivatives that have S595 mutated in a
35 manner that mimics S595 phosphorylation were generated. This was achieved by replacing S595 with glutamate or aspartate, both of which carry highly negative charged residues on top of their side chains to resemble a phosphate group on top of a serine. Additional eEF2 mutants have S595 or H715 replaced by alanine. H715A does not carry a diphthamide and therefore
40 cannot become ADP-ribosylated by toxins. In addition to carrying the described mutations, a HA-tag was placed upon the C-termini of the eEF2

derivatives to enable separation of recombinant eEF2 from non-mutated cellular eEF2, and to enable purification or specific detection of the recombinant eEF2 derivatives. As a control, recombinant wild-type (unmutated) eEF2 carrying a HA tag was also produced.

To test if the introduced mutations affect ADP-ribosylation of eEF2, expression plasmids encoding these mutants were transfected into MCF7 cells and extracts were generated 2 days later. To these extracts (containing normal cellular as well as recombinant mutated eEF2), beads were added that bind to the HA tag on the recombinant eEF2. Thereafter, Bio-NAD and toxin were added to enable toxin-catalyzed ADP-ribosylation reactions as described in Example 4. Recombinant HA-tag containing and possibly ADP-ribosylated eEF2 was separated from cellular proteins (including cellular eEF2) and enriched by a HA-bead affinity purification procedure, by applying magnetic anti-HA beads from Thermo Scientific according to the manufacturers protocol. This affinity purification procedure is well known to experts in the field. Subsequently, Western blots that detect HA-tagged protein, eEF2 and ADP ribosylation- (ADPR-)modified eEF2 were performed. The assay procedures are summarized in **Figure 7a**.

Figure 7b shows that these procedures enable the analyses of ADP-ribosylation of recombinant eEF2. HA-tag and eEF2 detecting Western blot analyses indicate that recombinant eEF2 becomes extracted by this procedure (positivity for eEF2 and HA antibodies at the correct molecular weight of eEF2). Signal positivity for ADP-ribosylated eEF2 from cells transfected with HA-tagged wildtype eEF2 indicated that this procedure or the C-terminal HA-tag do not interfere with the ADP-ribosylation reaction. In contrast to that, ADPR-signals were not observed in samples derived from cells that were transfected with HA-tagged H715A eEF2 (a mutation that lacks H715diphthamide), even though equal amounts of HA-tagged eEF2 were present (see HA-tag and eEF2 Westerns of the same sample). This demonstrated that the procedure enabled differentiation of eEF2 that is susceptible to ADP-ribosylation from ADPR-resistant eEF2. The result also proves that the preparations were not contaminated with cellular unmutated eEF2 as otherwise ADPR-signals would have been observed in the H715A mutated samples.

The application of this procedure for evaluation of susceptibility of eEF2 S595 mutants is shown in **Figure 7c**, again including HA-tagged wt- and H715A eEF2 as controls. All samples, i.e. controls as well as HA-eEF2 carrying the 595A, or 595D, or 595E mutations were loaded in similar amounts

(positivity for eEF2 and HA antibodies at the correct molecular weight of eEF2). Signal positivity for ADP-ribosylated eEF2 from cells transfected with HA-tagged wildtype eEF2 indicated that the procedure or the C-terminal HA-tag does not interfere with the ADP-ribosylation reaction. Lack of ADPR-signals in samples derived from cells that were transfected with the H715-diphthamide-negative HA-tagged H715A eEF2 demonstrated that the preparations were not contaminated with cellular (unmutated) eEF2.

The results of these analyses demonstrate that exchange of S595 to alanine (S595A), to glutamate (S595E) or to aspartate (S595D) interfered with ADP-ribosylation of eEF2, even though the mutated eEF2 was present in sufficient amounts (HA-tag signals) and the ADPR-reaction worked (signal positivity for HA-tagged wt eEF2). Thus, alterations at the S595 position of eEF2, including those that mimic S595 phosphorylation, interfere with toxin-mediated ADP-ribosylation at H715 diphthamide.

Conclusions: Interplay between reversible phosphorylation and irreversible ADP-ribosylation of eEF2

We have analyzed in MCF7 cells the influence on each other of ADP-ribosylation at the diphthamide of H715 and phosphorylation at T56 and S595 of eEF2.

Structural and biochemical analyses with eEF2 protein and extracts of cells exposed to kinase stimulators and ADP-ribosylating toxins demonstrate that S595 phosphorylation and H715-diphthamide ADP-ribosylation by *Pseudomonas* exotoxin and *Diphtheria* toxin interfere with each other. Also, eEF2 without diphthamide from homozygous DPH1 knockout cells cannot be ADP-ribosylated.

Inhibition of S595 phosphorylation upon H715 ADP-ribosylation can be explained by close proximity of S595 and diphthamide in the eEF2 structure, and by local structural changes upon H715 ADP-ribosylation. Conversely, inhibition of ADP-ribosylation upon phosphorylation is believed to result from the close proximity of S595 and diphthamide in the eEF2 structure, and by local structural changes upon S595 phosphorylation.

S595 phosphorylation was previously shown to modulate eEF2K-mediated T56 phosphorylation, followed by protein synthesis inhibition and induction of pro-survival pathways (Hizli et al. 2013, Leprivier et al. 2013). ADP-ribosylation of eEF2 at the close by position also inhibits protein

synthesis and induces a very similar transcriptional pathways, in particular a Jun/Fos/ATF3 response that is also associated with amino acid deprivation response.

5 Even though both eEF2 modifications appear to provide similar primary outcomes for cells, a major difference is that phosphorylation is short lived while ADP-ribosylation is stable. Thus, phosphorylation may provide a transient pro-survival 'emergency break' of translation under stress conditions, while ADP-ribosylation irreversibly blocks protein synthesis
10 leading to cell death.

Enzymes that ADP-ribosylate the diphthamide of eEF2 such as derivatives of PE and DT are currently applied as toxic payloads in targeted cancer therapy. S595 phosphorylation dependent modulation of ADP-ribosylation
15 susceptibility suggests that the 'eEF2 phosphorylation status' will correlate with drug response, and that inhibitors of kinases that phosphorylate eEF2 at S595 will enhance immunotoxin therapies and other targeted therapies.

20 Example 8: Generation and characterization of antibodies that specifically detect eEF2 that is phosphorylated at S595

Antibodies that specifically detect eEF2 with phosphorylation at S595, but
25 do not bind eEF2 that is not phosphorylated at S595, are highly desired for the analysis of the phosphorylation status of tumor cells in accordance with the invention. So far, such antibodies validated in extracts of tumor cells or on tumor tissues are not available. As proposed in the priority application EP 15159792.9, we applied a rabbit immunization and subsequent
30 B-cell-cloning procedure for the generation of antibodies that specifically bind eEF2 with a phosphorylation at S595.

Therefore, rabbits were immunized with a peptide spanning amino acids 583 - 606 of human eEF2 (583 - 606: VSEESNVLSLSK**pS**PNKHNRLY*Nle*KA, where **pS**
35 represents phosphoserine and *Nle* represents norleucine; SEQ ID NO:52), coupled to KLH. This peptide is phosphorylated at the serine residue that corresponds to the serine residue at position 595 of human eEF2. The methionine residue at position 604 of human eEF2 was replaced with norleucine to aid peptide purification.
40

After several rounds of immunization with adjuvant, B-cells expressing peptide-binding antibodies were isolated and enriched by magnetic-activated cell sorting (MACS) and single B-cells were sorted by FACS and subsequently converted to recombinant antibody clones via B-cell cloning and PCR-mediated V-region extraction (Seeber S. et al. PloS one; 2014;9(2):e86184 PMID:24503933).

The cloned and transiently expressed antibodies were analysed with phosphorylated peptide, with non-phosphorylated peptide of otherwise identical sequence, and with extracts of cells to analyze their selectivity for S595 phosphorylation. ELISA and Biacore analyses with the eEF2 peptide phosphorylated and non-phosphorylated at serine 595 were used to select antibody candidates. (This eEF2 peptide also contained the replacement norleucine residue at position 604.)

These analyses revealed that an antibody (clone 4F2) specifically binds to S595-phosphorylated eEF2 but not to eEF2 that is not phosphorylated at this position (Figure 8: Biacore data). Figure 8 demonstrates the specificity and functionality for detection of S595 phosphorylated eEF2 in Surface Plasmon Resonance Assays.

Western blot analyses (eEF2 detection in MCF-7 cell extracts) were performed to evaluate eEF2 specificity and to identify those clones that selectively recognize eEF2 (Figure 9). As shown by Western blot analysis the selected clone 4F2 only binds to eEF2 which is phosphorylated by stimulation. The sequences of the variable regions of the pS595-eEF2 specific antibody are shown below.

VH:

QSVEESGGRLVTPGTPLTLTCTVSGFSLNSYSIVWVRQAPGEGLEHIGFIDVDGAAHFASWVNGRFTISRTSTTV
DLKMTSLTAADTAAYFCARYGSGQLQLEVWGQGLVTVSS (SEQ ID NO:53)

VL:

DPVMTQTPSSTSAAVGGTVTINCQSSQNVYFNNYLSWFQQKPGQPPKLLIYRASTLASGVPSRFRKSGSGFTQFTL
TISGVQCDDAATYYCAGYYYNGGGIDTFVFGGGTEVVVKGD (SEQ ID NO:54)

The heavy chain CDRs/HVRs of clone 4F2 are variously defined according to Kabat, Chothia and AbM as follows:

CDRH1: GFSLNSYSIV (AbM; SEQ ID NO:55)
 GFSLNS (Chothia; SEQ ID NO:56)
 SYSIV (Kabat; SEQ ID NO:57)

5 CDRH2: FIDVDGAAHFASWVNG (Kabat; SEQ ID NO:58)
 FIDVDGAAH (AbM / Chothia; SEQ ID NO:59)

CDRH3: YGSGLQLEV (all; SEQ ID NO:60)

10 CDRL1: QSSQNVYFNYYLS (all; SEQ ID NO:61)

CDRL2: RASTLAS (all; SEQ ID NO:62)

CDRL3: AGYYNNGGIDTFV (all; SEQ ID NO:63).

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While there are shown and described presently preferred embodiments of the invention, it is to be distinctly understood that the invention is not limited thereto but may be otherwise variously embodied and practised within the scope of the following claims.

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- 20 All documents cited in the text (whether included in this list of selected references or not) are incorporated by reference herein in their entirety and for all purposes.

CLAIMS

1. A method for assessing resistance or non-resistance of diseased cells in a patient to treatment with a NAD(+)-diphthamide ADP-ribosyltransferase, the method comprising assaying for serine phosphorylation of eEF2 protein in a sample containing diseased cells, wherein serine phosphorylation of the eEF2 protein is indicative that the diseased cells are resistant to treatment with a NAD(+)-diphthamide ADP-ribosyltransferase.
2. The method of claim 1, which further includes a step of selecting the patient for treatment with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to the diseased cells of the patient if the diseased cells of the sample are assessed to be non-resistant to NAD(+)-diphthamide ADP ribosyltransferase.
3. The method of claim 1 or claim 2, which further includes a step of deselecting the patient for treatment with a NAD(+)-diphthamide ADP ribosyltransferase if the diseased cells are assessed to be resistant to NAD(+)-diphthamide ADP ribosyltransferase.
4. The method of claim 2 or claim 3, which further includes a step, following selection of the patient for treatment, of treating the patient with the targeted therapeutic agent.
5. A method for selecting and/or deselecting a patient for treatment with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to diseased cells of the patient, the method comprising:
 - (i) assaying for serine phosphorylation of eEF2 protein in a sample containing the diseased cells from the patient; and
 - (ii) (a) selecting the patient for treatment with the targeted therapeutic agent if the assay is negative for serine phosphorylation of the eEF2 protein; and/or
 - (ii) (b) deselecting the patient for treatment with the targeted therapeutic agent if the assay is positive for serine phosphorylation of the eEF2 protein.
6. The method of claim 4, further comprising a step, following selection in (ii) (a) of the patient for treatment, of treating the patient with the targeted therapeutic agent.

7. A method for treating a patient having a condition that is treatable by cytotoxic activity targeted to diseased cells of the patient, the method comprising:

assaying a sample containing diseased cells from a patient for serine phosphorylation of eEF2 protein; and

treating a patient in whose sample the assay is negative for serine phosphorylation of eEF2 protein with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to the diseased cells of the patient.

8. A method for treating a patient having a condition that is treatable by cytotoxic activity targeted to diseased cells of the patient, the method comprising:

assaying for serine phosphorylation of eEF2 protein in a sample containing the diseased cells from the patient;

assessing resistance or non-resistance of the diseased cells to treatment with a NAD(+)-diphthamide ADP-ribosyltransferase, wherein serine phosphorylation of the eEF2 protein is indicative that the diseased cells are resistant to treatment with a NAD(+)-diphthamide ADP-ribosyltransferase; and

treating a patient whose diseased cells are assessed to be non-resistant with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to the diseased cells of the patient.

9. A method for treating a patient having a condition that is treatable by cytotoxic activity targeted to diseased cells of the patient, the method comprising:

treating the patient with a targeted therapeutic agent comprising a NAD(+)-diphthamide ADP ribosyltransferase coupled to a cell-binding agent targeted to the diseased cells of the patient, wherein the patient is selected for treatment on the basis of a negative assay result for serine phosphorylation of eEF2 protein in a sample containing diseased cells from the patient.

10. The method of any one of claims 1-9, wherein the assay for serine phosphorylation of eEF2 protein is performed with an antibody that selectively binds to eEF2 or a fragment thereof that is phosphorylated at the S595 residue.

11. The method of claim 10, wherein the binding affinity of the antibody for eEF2 or a fragment thereof that is phosphorylated at the S595 residue is at least 10-fold higher than the binding affinity for eEF2 or a fragment thereof that is non-phosphorylated at the S595 residue.

12. The method of claim 11, wherein the antibody substantially does not bind to eEF2 or a fragment thereof that is non-phosphorylated at the S595 residue.

13. The method of any one of claims 10-12, wherein the antibody binds to eEF2 or a fragment thereof that is phosphorylated at the S595 residue with a K_D of 100 nM or less.

14. The method of any one of claims 10-13, wherein the antibody is a monoclonal antibody having the heavy chain variable domain sequence of SEQ ID NO:53, or a heavy chain variable domain sequence having at least 80%, 85%, 90%, 95%, 98% or 99% identity to SEQ ID NO:53.

15. The method of any one of claims 10-14, wherein the antibody is a monoclonal antibody having the light chain variable domain sequence of SEQ ID NO:54, or a light chain variable domain sequence having at least 80%, 85%, 90%, 95%, 98% or 99% identity to SEQ ID NO:54.

16. The method of any one of claims 10-15, wherein the antibody is a monoclonal antibody having the CDR-H1 sequence of SEQ ID NO:55, 56 or 57, or said CDR-H1 sequence with one or more amino acid insertions, deletions and/or substitutions.

17. The method of any one of claims 10-16, wherein the antibody is a monoclonal antibody having the CDR-H2 sequence of SEQ ID NO:58 or 59, or said CDR-H2 sequence with one or more amino acid insertions, deletions and/or substitutions.

18. The method of any one of claims 10-17, wherein the antibody is a monoclonal antibody having the CDR-H3 sequence of SEQ ID NO:60, or said CDR-H3 sequence with one or more amino acid insertions, deletions and/or substitutions.

19. The method of any one of claims 10-18, wherein the antibody is a monoclonal antibody having the CDR-L1 sequence of SEQ ID NO:61, or said CDR-L1 sequence with one or more amino acid insertions, deletions and/or substitutions.

20. The method of any one of claims 10-19, wherein the antibody is a monoclonal antibody having the CDR-L2 sequence of SEQ ID NO:62, or said CDR-H2 sequence with one or more amino acid insertions, deletions and/or substitutions.

21. The method of any one of claims 10-20, wherein the antibody is a monoclonal antibody having the CDR-L3 sequence of SEQ ID NO:63, or said CDR-L3 sequence with one or more amino acid insertions, deletions and/or substitutions.

22. The method of any one of claims 10-21, wherein the antibody is a monoclonal antibody having the heavy chain complementarity-determining region (CDR) sequence H3 of SEQ ID NO:60.

23. The method of claim 22, wherein the antibody has the heavy chain CDR sequences H2 of SEQ ID NO:58 or 59 and H3 of SEQ ID NO:60.

24. The method of claim 23, wherein the antibody has the heavy chain CDR sequences H1 of SEQ ID NO:55, 56 or 57, H2 of SEQ ID NO: 58 or 59 and H3 of the SEQ ID NO:60.

25. The method of any one of claims 10-24, wherein the antibody is a monoclonal antibody having the light chain complementarity-determining region (CDR) sequence L3 of SEQ ID NO:63.

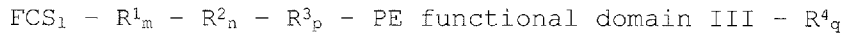
26. The method of claim 25, wherein the antibody has the light chain CDR sequences L2 and L3 of SEQ ID NOs:62 and 63.

27. The method of claim 26, wherein the antibody has the light chain CDR sequences L1, L2 and L3 of SEQ ID NOs:61 to 63.

28. The method of any one of claims 10-27, wherein the antibody is a monoclonal antibody comprising the CDR sequences H1 of SEQ ID NO:55, 56 or 57, H2 of SEQ ID NO:58 or 59, and H3 of SEQ ID NO:60 and the CDR L1-L3 sequences of the SEQ ID NOs:61 to 63.

29. The method of any one of claims 10-28, wherein the antibody is a monoclonal antibody comprising the heavy chain variable domain sequence shown in SEQ ID NO:53 and the light chain variable domain sequence shown in SEQ ID NO:54.

30. The method of any one of claims 1-9, wherein the assay for serine phosphorylation of eEF2 protein is performed using a non site-specific anti-phosphoserine antibody.
31. The method of any one of claims 10-30, wherein the assay comprises subjecting an extract of the sample to chromatography and contacting one or more chromatography fractions with said antibody.
32. The method of any one of claims 10-30, wherein the assay comprises subjecting an extract of the sample to electrophoresis and contacting the electrophoresis gel or a blot thereof with said antibody.
33. The method of any one of claims 10-30, wherein the assay comprises subjecting an extract of the sample to a sandwich assay comprising said antibody as a capture antibody or detection antibody.
34. The method of claim 33, wherein the assay is an ELISA assay.
35. The method of any one of claims 10-30, wherein the assay comprises subjecting an extract of the sample to a dipstick test comprising said antibody.
36. The method according to any one of claims 10-35, wherein said antibody is labelled with a detectable label.
37. The method of any one of claims 2-36, wherein the NAD(+)-diphthamide ADP ribosyltransferase of the targeted therapeutic agent is a PE toxin, a DT toxin or a cholix toxin.
38. The method of claim 37, wherein the NAD(+)-diphthamide ADP ribosyltransferase of the targeted therapeutic agent is a PE toxin.
39. The method of any one of claims 36-38, wherein the PE toxin has a polypeptide sequence comprising a PE functional domain III having at least 50% amino acid sequence identity over the full length of residues 395-601 of SEQ ID NO:1, wherein the PE toxin has cytotoxic activity when introduced into a mammalian cell.
40. The method of any one of claims 37-39, wherein the PE toxin has the following structure:



wherein:

l, m, n, p and q are each, independently, 0 or 1;

FCS is a furin-cleavable sequence, preferably (i) R-H-R-Q-P-R-G-W-E-Q-L (SEQ ID NO: 6) or a truncated version thereof containing R-Q-P-R (SEQ ID NO: 28), optionally R-Q-P-R (SEQ ID NO: 28), R-H-R-Q-P-R-G-W (SEQ ID NO: 29), R-H-R-Q-P-R-G-W-E (SEQ ID NO: 30), H-R-Q-P-R-G-W-E-Q (SEQ ID NO: 31), or R-Q-P-R-G-W-E (SEQ ID NO: 32); or (ii) R-H-R-S-K-R-G-W-E-Q-L (SEQ ID NO: 18) or a truncated version thereof containing R-S-K-R (SEQ ID NO: 33), optionally R-S-K-R (SEQ ID NO: 33), R-H-R-S-K-R-G-W (SEQ ID NO: 34), H-R-S-K-R-G-W-E (SEQ ID NO: 35), R-S-K-R-G-W-E-Q-L (SEQ ID NO: 36), H-R-S-K-R-G-W-E-Q-L (SEQ ID NO: 37), or R-H-R-S-K-R (SEQ ID NO: 38), wherein the glutamic acid residue corresponding to position 282 of the native PE sequence (where present) is optionally replaced by another residue, preferably glycine, serine, alanine or glutamine;

R¹ is a linker sequence of 1 to 10 amino acids, preferably GGS or GGSGGS (SEQ ID NO: 7);

R² is one or more consecutive amino acid residues of residues 285-364 of SEQ ID NO:1, in which any one or more of residues E285, P290, L294, L297, Y298, L299, R302, R313, N314, P319, D324, E327, E331 and Q332, where present, is/are optionally independently replaced by another amino acid, preferably glycine, serine, alanine or glutamine;

R³ is one or more consecutive amino acid residues of residues 365-394 of SEQ ID NO:1;

PE functional domain III comprises residues 395-613 of SEQ ID NO:1 in which:

- (a) some or all of residues 602-608 are optionally deleted, and
- (b) residues 609-613 are optionally replaced by another ER localisation sequence, preferably KDEL (SEQ ID NO: 9), REDL (SEQ ID NO:10), RDEL (SEQ ID NO:11) or KEDLK (SEQ ID NO:12), and
- (c) any one or more of residues D403, D406, R412, E420, R421, L422, L423, A425, R427, L429, E431, R432, Y439, H440, F443, L444, A446, A447, I450, R456, R458, D461, 463-519 (preferably D463, R467, L477, Y481, R490, R494, R505, R513 and/or L516), E522, R538, E548, R551, L552, T554, I555, L556, W558, R563, R576, D581, D589, K590, Q592, L597 and (where present) K606 is/are optionally independently replaced by another amino acid, preferably glycine, serine, alanine or glutamine, or histidine in the case of L477;

R⁴ is one or more (preferably 1 or 2) additional ER localisation sequences, preferably REDLK (SEQ ID NO: 8), KDEL (SEQ ID NO: 9), REDL (SEQ ID NO:10), RDEL (SEQ ID NO:11) or KEDLK (SEQ ID NO:12).

41. The method of claim 40, wherein l is 1.

42. The method of claim 40 or claim 41, wherein m is 1.

43. The method of any one of claims 40-42, wherein n is 0.

44. The method of any one of claims 40-43, wherein p is 0.

45. The method of any one of claims 40-44, wherein q is 0.

46. The method of any one of claims 40-45, wherein the PE functional domain III includes the combination of mutations

R427A/F443A/L477H/R494A/R505A/L552E, or the combination of mutations

R427A/R456A/D463A/R467A/R490A/R505A/R538A, or the combination of mutations

R427A/F443A/R456A/D463A/R467A/L477H/R490A/R494A/R505A/R538A/L552E.

47. The method of any one of claims 40-46, wherein the PE toxin comprises the amino acid sequence of SEQ ID NO:64 or SEQ ID NO:65.

48. The method of claim 47, wherein the amino acid sequence of SEQ ID NO:64 or SEQ ID NO:65 is fused to the C-terminal end of the amino acid sequence of SEQ ID NO:66.

49. The method of claim 37, wherein the DT toxin has a polypeptide sequence comprising a DT functional domain I having at least 50% amino acid sequence identity over the full length of residues 1-191 of SEQ ID NO:3, wherein the DT toxin has cytotoxic activity when introduced into a mammalian cell.

50. The method of claim 37, wherein the cholix toxin has a polypeptide sequence comprising a cholix toxin functional domain III having at least 50% amino acid sequence identity over the full length of residues 424-628 of SEQ ID NO:4, wherein the cholix toxin has cytotoxic activity when introduced into a mammalian cell.

51. The method of any one of claims 2-50, wherein the targeted therapeutic agent comprises a NAD(+)-diphthamide ADP ribosyltransferase coupled to the cell-binding agent as a fusion polypeptide.
52. The method of claim 51 wherein the NAD(+)-diphthamide ADP ribosyltransferase is directly coupled to the cell-binding agent as a fusion polypeptide.
53. The method of any one of claims 2-52, wherein the cell-binding agent is an antibody.
54. The method of claim 53, wherein the antibody is an antigen-binding antibody fragment.
55. The method of claim 53 or claim 54, wherein the antibody is directed against a tumour-associated antigen.
56. The method of any one of claims 1-55, wherein the diseased cells are pre-cancer, cancer or tumour cells, virally-infected cells or autoimmune effector cells.
57. The method of claim 56, wherein the diseased cells are pre-cancer, cancer or tumour cells.
58. The method of any one of claims 7-57, wherein the condition is a pre-cancer, cancer, tumour, viral infection or autoimmune disease.
59. The method of claim 58, wherein the condition is a pre-cancer, cancer or tumour.
60. The method of any one of claims 1-59, wherein the patient is human.
61. A NAD(+)-diphthamide ADP ribosyltransferase for use in a method of medical treatment of a patient from whom a sample containing diseased cells has given a negative result in an assay for serine phosphorylation of eEF2 protein.
62. A NAD(+)-diphthamide ADP ribosyltransferase for use in a method of medical treatment of a patient from whom a sample containing diseased cells has been assayed for serine phosphorylation of eEF2 protein and assessed as non-resistant to NAD(+)-diphthamide ADP ribosyltransferase treatment.

63. The NAD(+)-diphthamide ADP ribosyltransferase for use of claim 61 or claim 62, wherein the assay for serine phosphorylation of eEF2 protein was performed with an antibody that selectively binds to eEF2 or a fragment thereof that is phosphorylated at the S595 residue.

64. The NAD(+)-diphthamide ADP ribosyltransferase for use of claim 63, wherein the antibody is as defined in any one of claims 11 to 29.

65. The NAD(+)-diphthamide ADP ribosyltransferase for use of claim 61 or claim 62, wherein the assay for serine phosphorylation of eEF2 protein was performed using a non site-specific anti-phosphoserine antibody.

66. The NAD(+)-diphthamide ADP ribosyltransferase for use of any one of claims 61 to 65, wherein the NAD(+)-diphthamide ADP ribosyltransferase is as defined in any one of claims 37-50.

67. The NAD(+)-diphthamide ADP ribosyltransferase for use of any one of claims 61 to 66, wherein the NAD(+)-diphthamide ADP ribosyltransferase is coupled to a cell-binding agent targeted to diseased cells of the patient.

68. The NAD(+)-diphthamide ADP ribosyltransferase for use of any one of claims 61 to 67, wherein the NAD(+)-diphthamide ADP ribosyltransferase is coupled to the cell-binding agent as a fusion polypeptide.

69. The NAD(+)-diphthamide ADP ribosyltransferase for use of claim 68, wherein the NAD(+)-diphthamide ADP ribosyltransferase is directly coupled to the cell-binding agent as a fusion polypeptide.

70. The NAD(+)-diphthamide ADP ribosyltransferase for use of any one of claims 67-69, wherein the cell-binding agent is an antibody.

71. The NAD(+)-diphthamide ADP ribosyltransferase for use of claim 70, wherein the antibody is an antigen-binding antibody fragment.

72. The NAD(+)-diphthamide ADP ribosyltransferase for use of claim 70 or claim 71, wherein the antibody is directed against a tumour-associated antigen.

73. The NAD(+)-diphthamide ADP ribosyltransferase for use of any one of claims 61 to 72, wherein the diseased cells are pre-cancer, cancer or tumour cells, virally-infected cells or autoimmune effector cells.

74. The NAD(+)-diphthamide ADP ribosyltransferase for use of claim 73, wherein the diseased cells are pre-cancer, cancer or tumour cells.

75. The NAD(+)-diphthamide ADP ribosyltransferase for use of any one of claims 61 to 74, which is for use in the treatment of a pre-cancer, cancer, tumour, viral infection or autoimmune disease.

76. The NAD(+)-diphthamide ADP ribosyltransferase for use of claim 75, which is for use in the treatment of a pre-cancer, cancer or tumour.

77. The NAD(+)-diphthamide ADP ribosyltransferase for use of any one of claims 61 to 76, which is for use in the treatment of a human patient.

78. A NAD(+)-diphthamide ADP ribosyltransferase for use in a method of medical treatment of a patient having a condition that is treatable by cytotoxic activity targeted to diseased cells of the patient, wherein the method is as defined in any one of claims 4, 6 and 8 to 60.

79. A monoclonal antibody that selectively binds to eEF2 or a fragment thereof that is phosphorylated at the S595 residue.

80. The monoclonal antibody of claim 79, having a binding affinity for eEF2 or a fragment thereof that is phosphorylated at the S595 residue at least 10-fold higher than the binding affinity for eEF2 or a fragment thereof that is non-phosphorylated at the S595 residue.

81. The monoclonal antibody of claim 79 or claims 80, which substantially does not bind to eEF2 or a fragment thereof that is non-phosphorylated at the S595 residue.

82. The monoclonal antibody of any one of claims 79 to 81, wherein the antibody binds to eEF2 or a fragment thereof that is phosphorylated at the S595 residue with a K_D of 100 nM or less.

83. The monoclonal antibody of any one of claims 79 to 82, having the heavy chain variable domain sequence of SEQ ID NO:53, or a heavy chain

variable domain sequence having at least 80%, 85%, 90%, 95%, 98% or 99% identity to SEQ ID NO:53.

84. The monoclonal antibody of any one of claims 79 to 83, having the light chain variable domain sequence of SEQ ID NO:54, or a light chain variable domain sequence having at least 80%, 85%, 90%, 95%, 98% or 99% identity to SEQ ID NO:54.

85. The monoclonal antibody of any one of claims 79 to 82, having the CDR-H1 sequence of SEQ ID NO:55, 56 or 57, or said CDR-H1 sequence with one or more amino acid insertions, deletions and/or substitutions.

86. The monoclonal antibody of any one of claims 79 to 85, having the CDR-H2 sequence of SEQ ID NO:58 or 59, or said CDR-H2 sequence with one or more amino acid insertions, deletions and/or substitutions.

87. The monoclonal antibody of any one of claims 79 to 86, having the CDR-H3 sequence of SEQ ID NO:60, or said CDR-H3 sequence with one or more amino acid insertions, deletions and/or substitutions.

88. The monoclonal antibody of any one of claims 79 to 87, having the CDR-L1 sequence of SEQ ID NO:61, or said CDR-L1 sequence with one or more amino acid insertions, deletions and/or substitutions.

89. The monoclonal antibody of any one of claims 79 to 88, having the CDR-L2 sequence of SEQ ID NO:62, or said CDR-H2 sequence with one or more amino acid insertions, deletions and/or substitutions.

90. The monoclonal antibody of any one of claims 79 to 89, having the CDR-L3 sequence of SEQ ID NO:63, or said CDR-L3 sequence with one or more amino acid insertions, deletions and/or substitutions.

91. The monoclonal antibody of any one of claims 79 to 90, having the heavy chain complementarity-determining region (CDR) sequence H3 of SEQ ID NO:60.

92. The monoclonal antibody of claim 91, having the heavy chain CDR sequences H2 of SEQ ID NO:58 or 59 and H3 of SEQ ID NO:60.

93. The monoclonal antibody of claim 92, having the heavy chain CDR sequences H1 of SEQ ID NO:55, 56 or 57, H2 of SEQ ID NO: 58 or 59 and H3 of the SEQ ID NO:60.

94. The monoclonal antibody of any one of claims 79 to 93, having the light chain complementarity-determining region (CDR) sequence L3 of SEQ ID NO:63.

95. The monoclonal antibody of claim 94, having the light chain CDR sequences L2 and L3 of SEQ ID NOs:62 and 63.

96. The monoclonal antibody of claim 95, having the light chain CDR sequences L1, L2 and L3 of SEQ ID NOs:61 to 63.

97. The monoclonal antibody of any one of claims 79 to 96, comprising the CDR sequences H1 of SEQ ID NO:55, 56 or 57, H2 of SEQ ID NO:58 or 59, and H3 of SEQ ID NO:60 and the CDR L1-L3 sequences of the SEQ ID NOs:61 to 63.

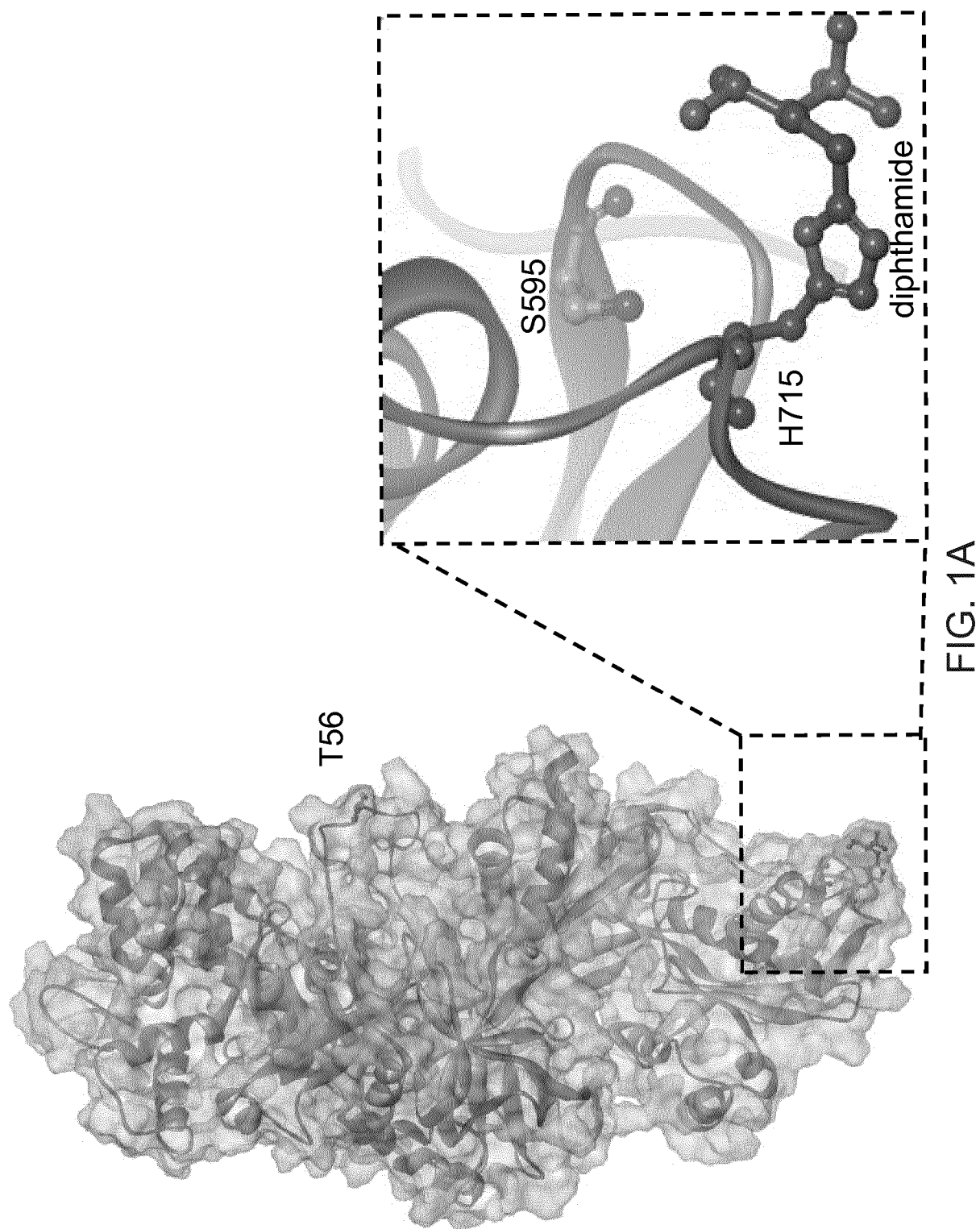
98. The monoclonal antibody of any one of claims 79 to 97, comprising the heavy chain variable domain sequence shown in SEQ ID NO:53 and the light chain variable domain sequence shown in SEQ ID NO:54.

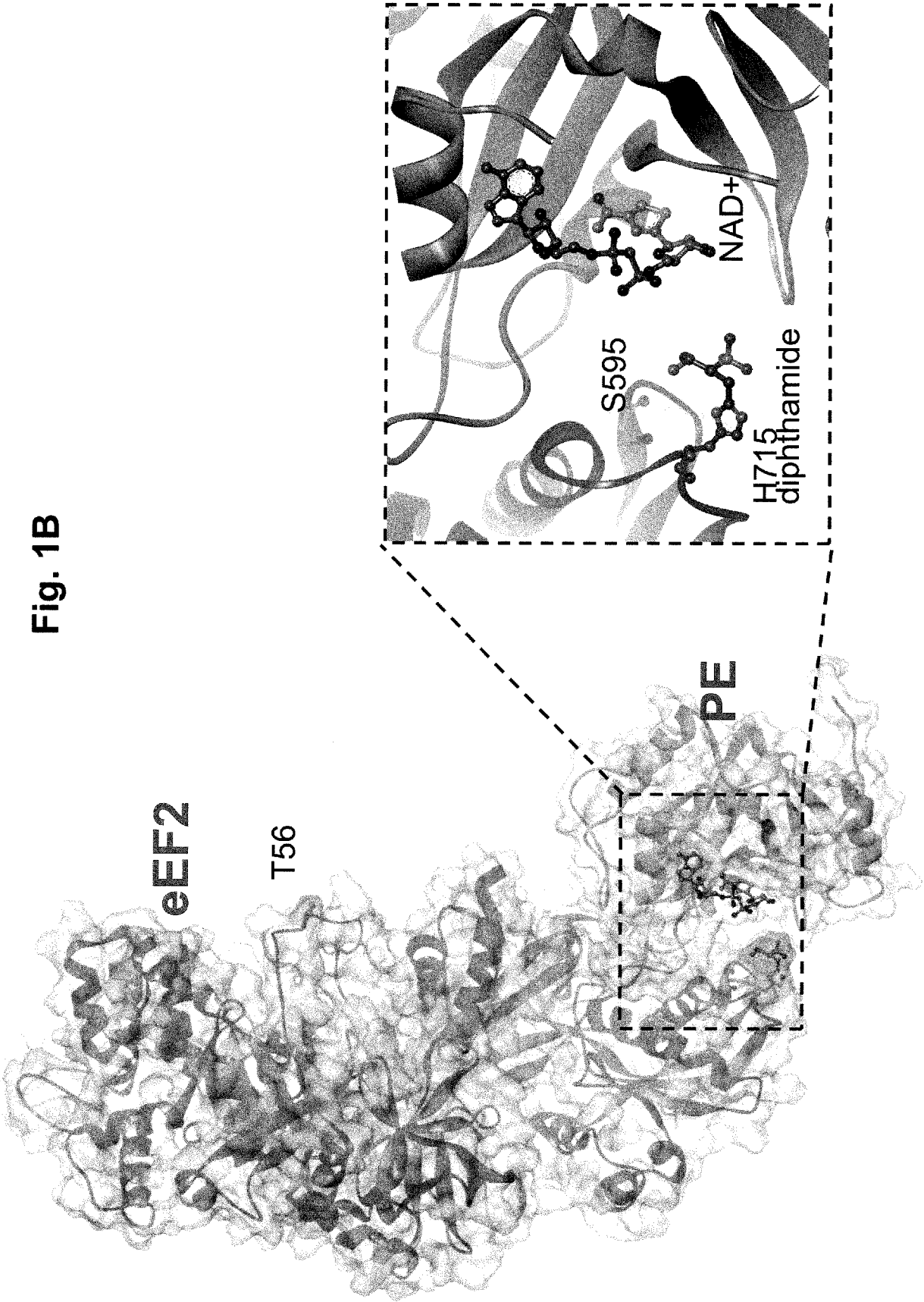
99. The monoclonal antibody according to any one of claims 79 to 98, which is labelled with a detectable label.

100. The monoclonal antibody according to claim 99, wherein the detectable label is an enzyme, a fluorescent label, a radiolabel, an electroluminescent label or biotin.

101. Use of the monoclonal antibody according to any one of claims 79 to 100 in an *in vitro* method of assessing resistance or non-resistance of a cell population to NAD(+)-diphthamide ADP-ribosyltransferase treatment.

102. Use of the monoclonal antibody according to any one of claims 79 to 100 in the method of any one of claims 1 to 60.





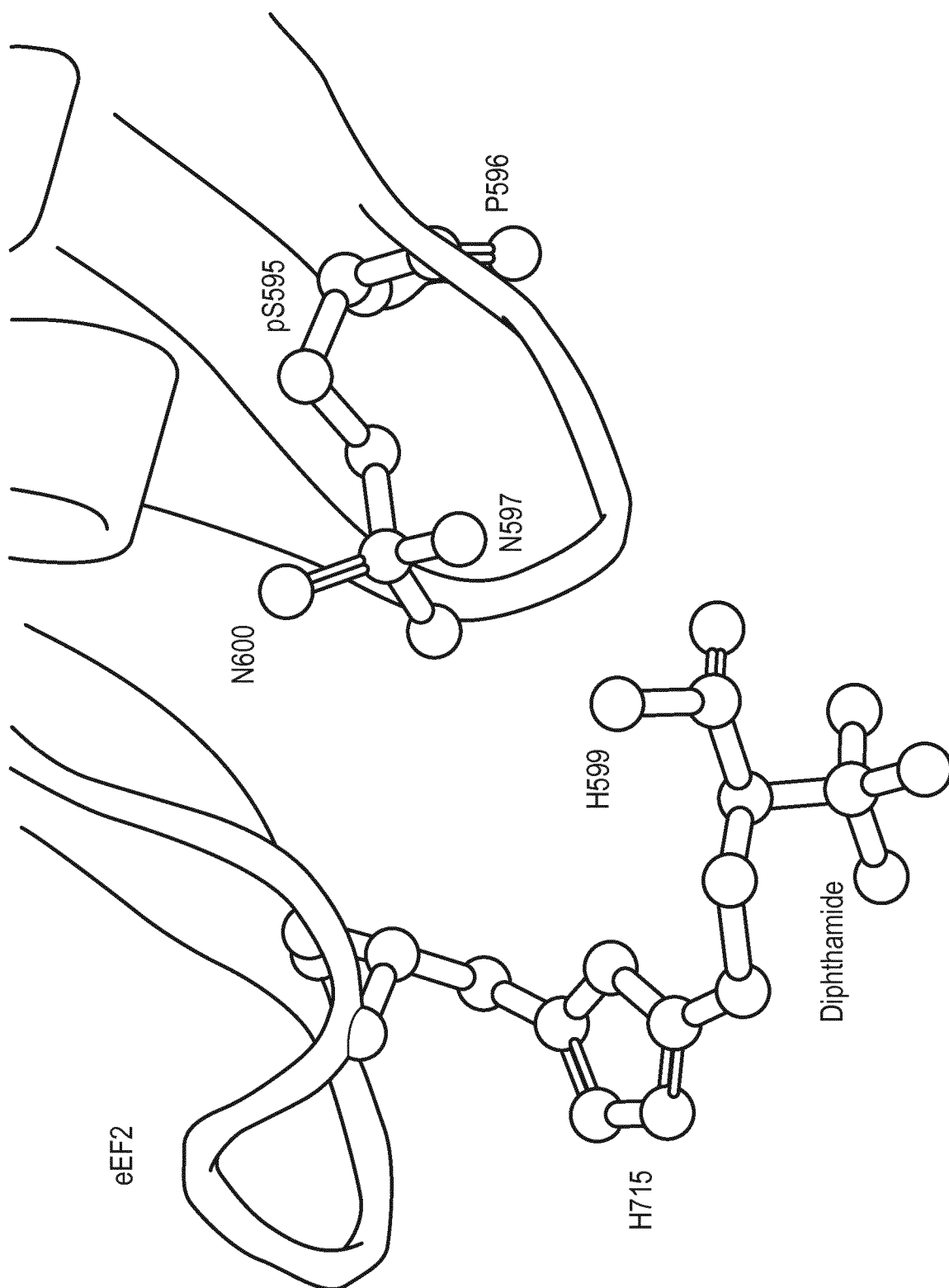


FIG. 1C

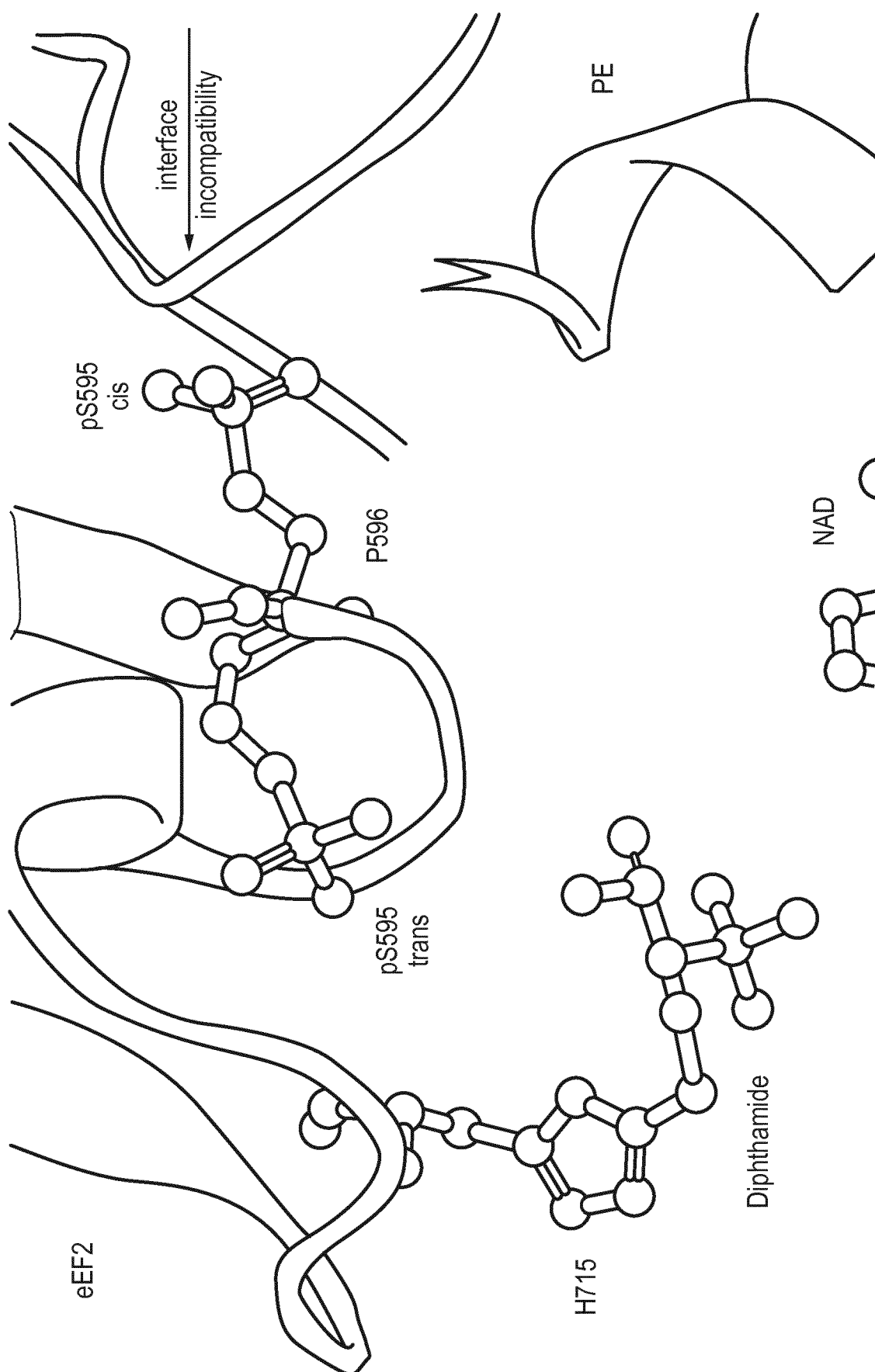


FIG. 1D

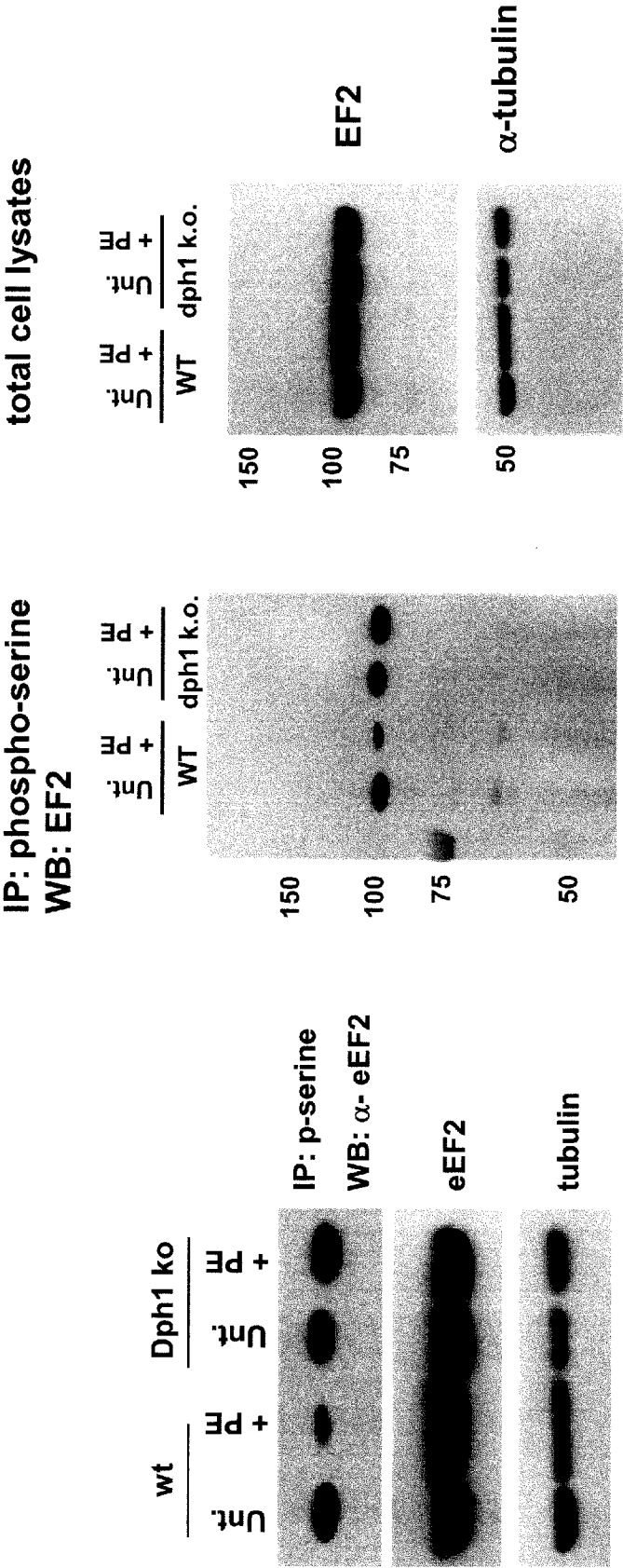


Figure 2

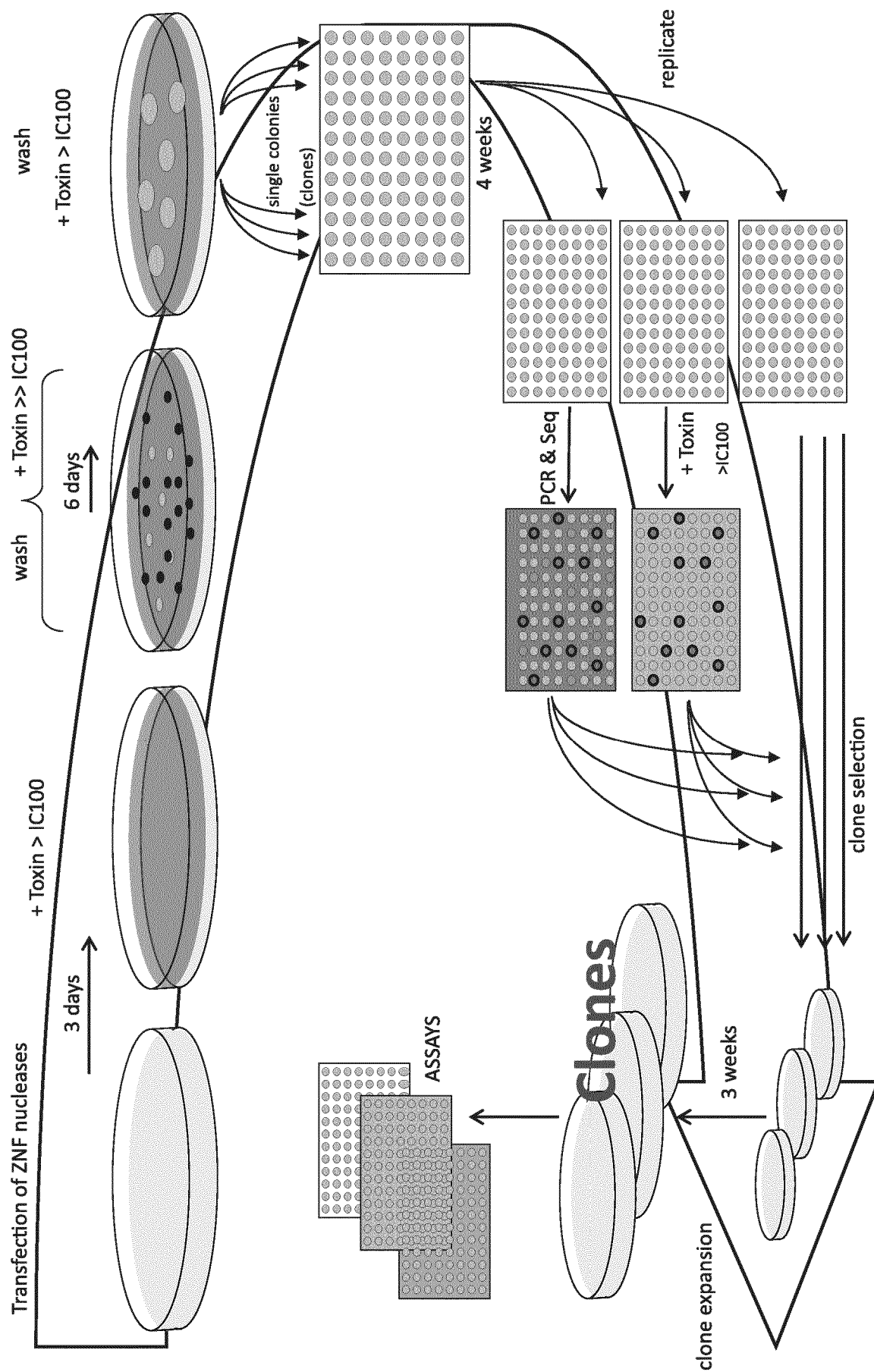


FIG. 3A

[illegible]

Fig. 3B

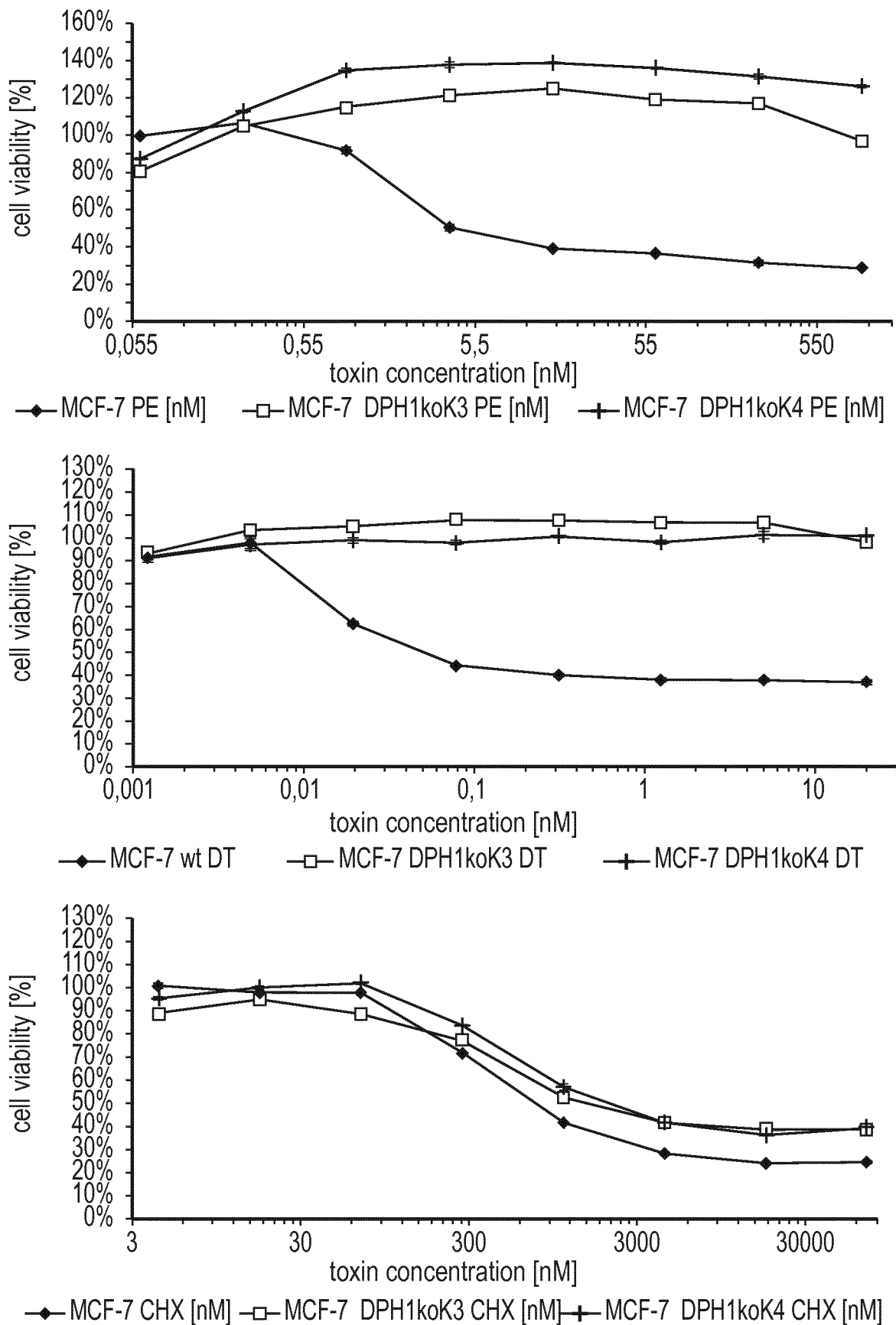


FIG. 3C

Fig. 4a

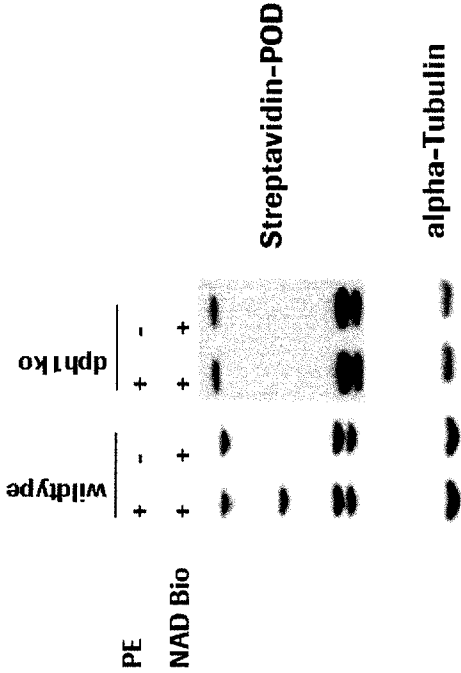


Fig. 4b

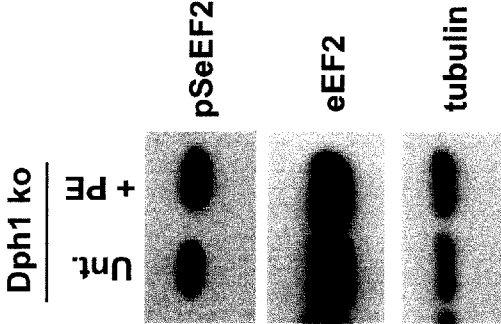


Fig. 4c

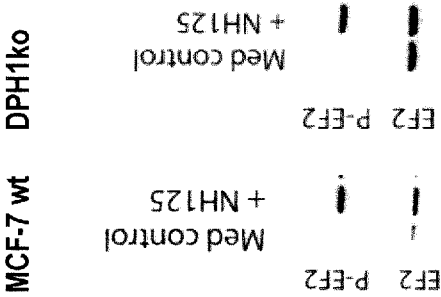


Fig. 5A

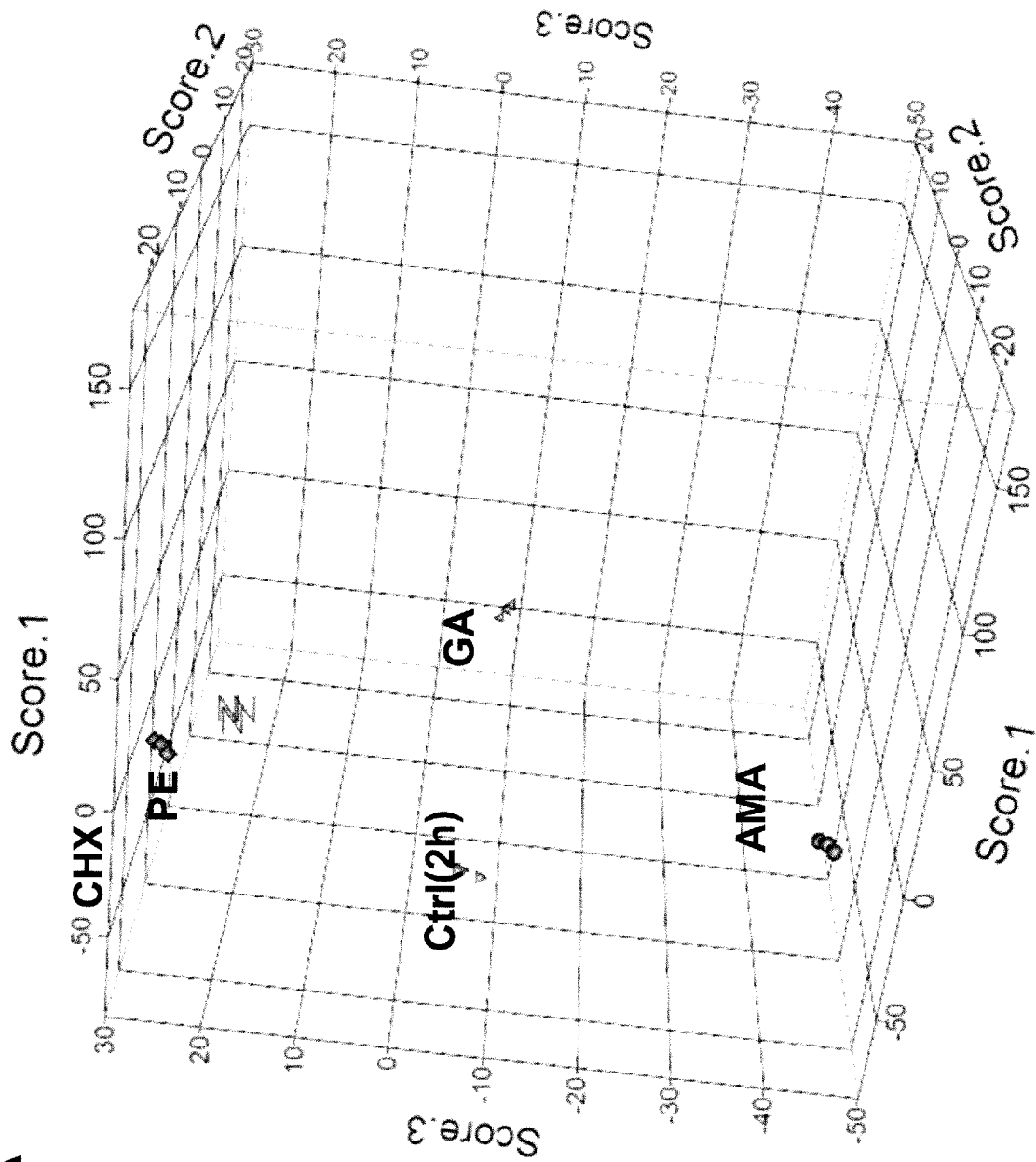


Fig. 5B

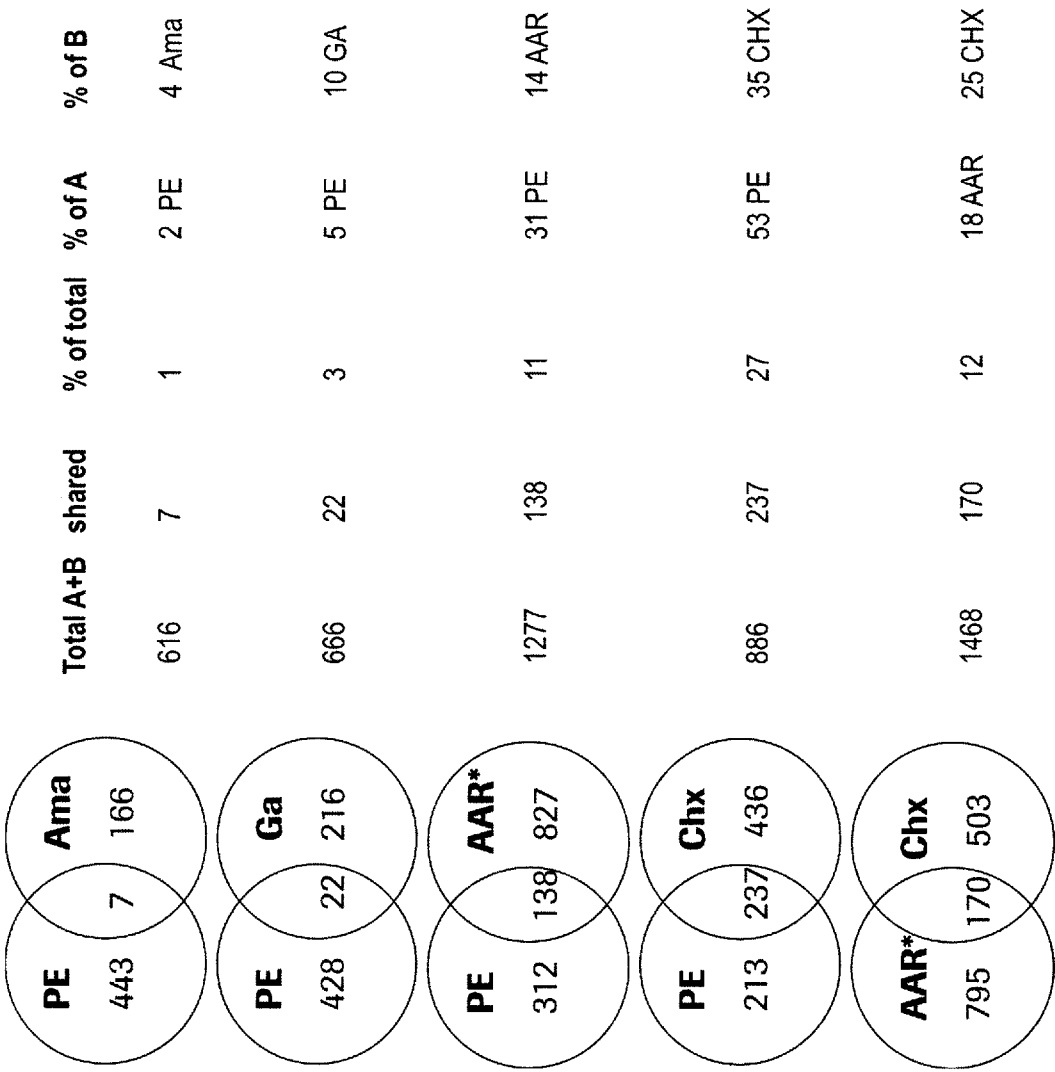


Figure 6

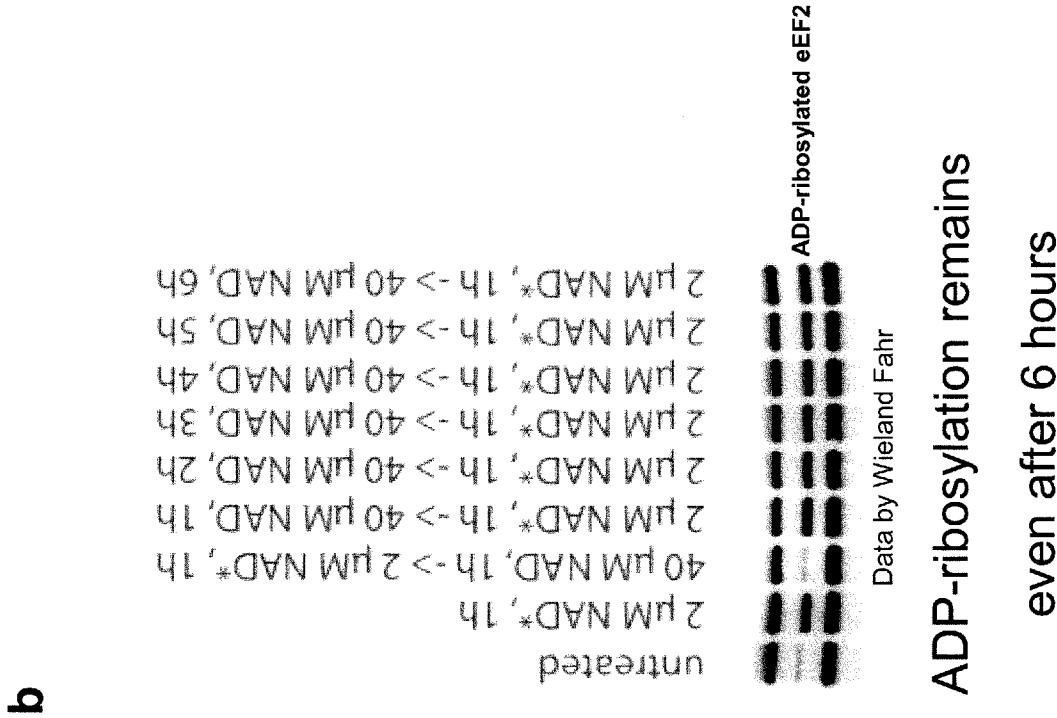


Figure 7A

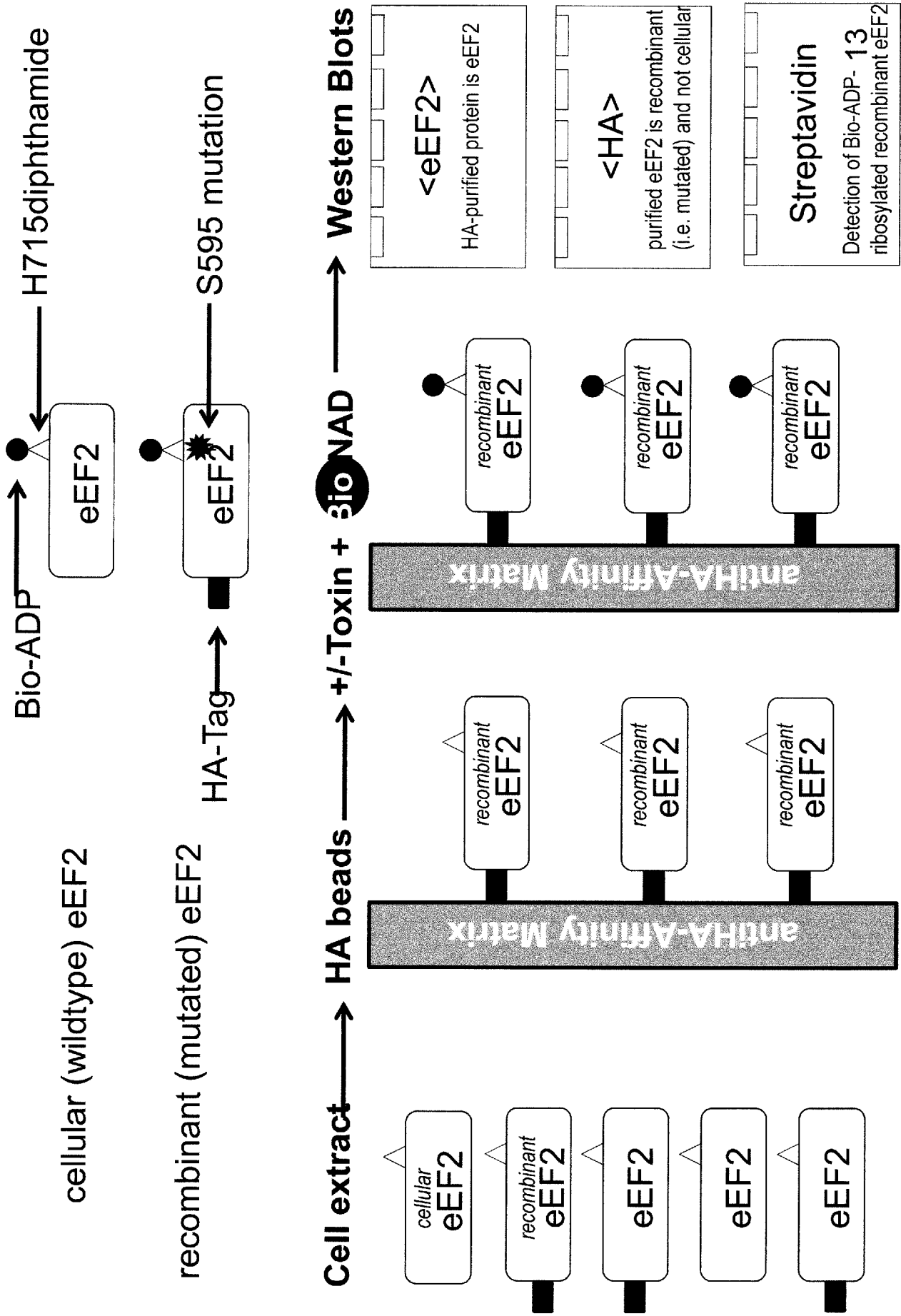


Figure 7B

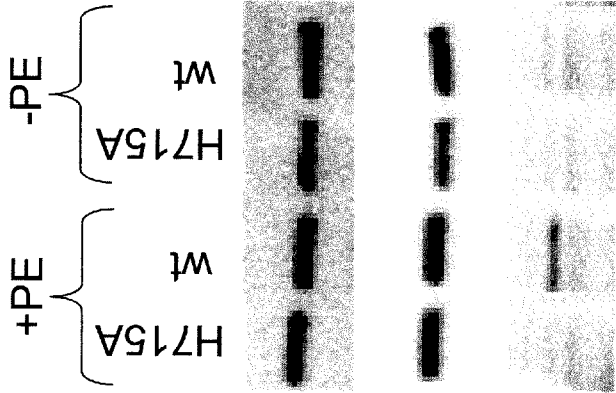


Figure 7C

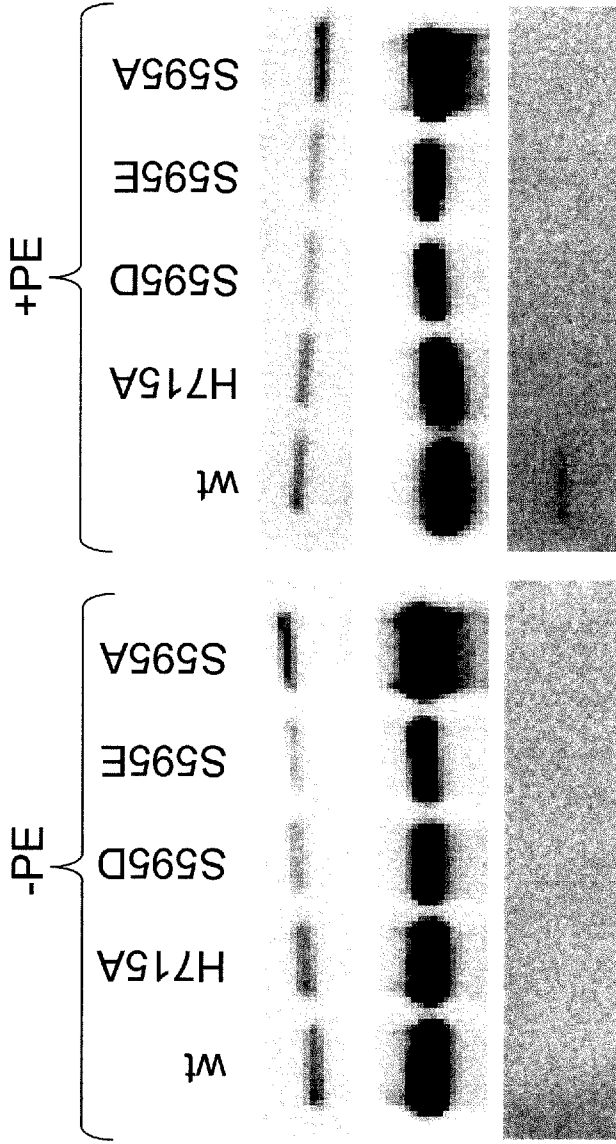
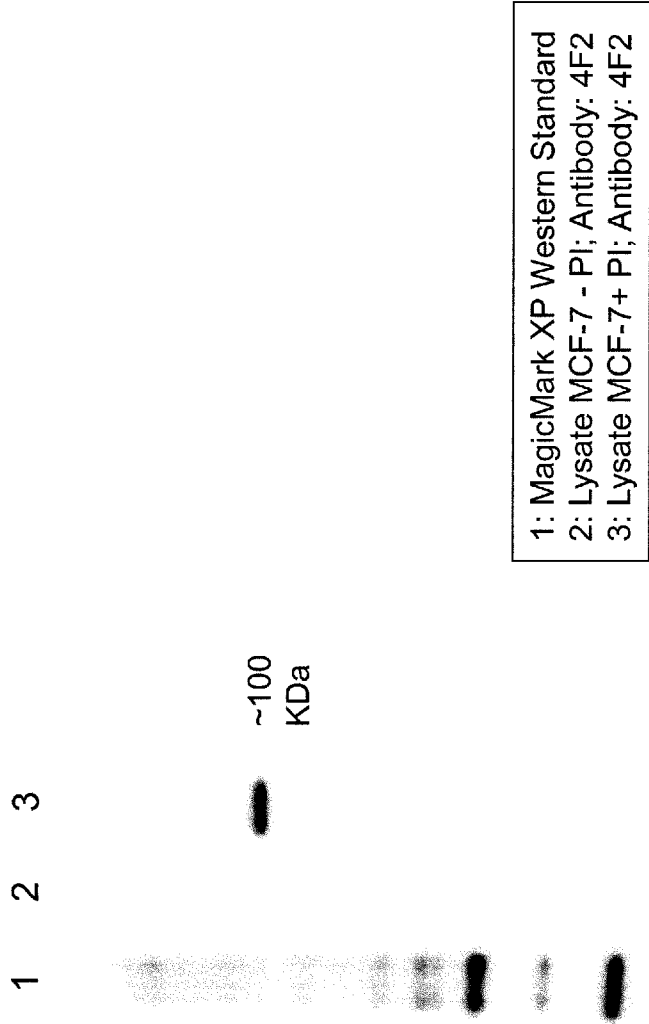


Figure 8:

clone	peptide/ analyte	BL [RU]	SL [RU]	k_d [1/s]	$t/2^{diss}$ [min]	T [°C]	CL [RU]	MR [-]
4F2	eEF2 (583 – 606)	3	3	n.d.	n.d.	25	468	0.0
4F2	eEF2 (583 – 606)-pSer595	171	160	2.45E-04	47	25	450	0.9

Figure 9:



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/056028

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	H. WEI ET AL: "A Modified Form of Diphthamide Causes Immunotoxin Resistance in a Lymphoma Cell Line with a Deletion of the WDR85 Gene", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 288, no. 17, 26 April 2013 (2013-04-26), pages 12305-12312, XP055212219, ISSN: 0021-9258, DOI: 10.1074/jbc.M113.461343	7-38, 51-78
Y	abstract page 12305, column 2, paragraph 2 page 12311, column 2, paragraph 3 ----- -/--	1-6



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

11 May 2016

Date of mailing of the international search report

13/07/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

van der Kooij, M

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/056028

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/040141 A1 (US HEALTH [US]; PASTAN IRA H [US]; ONDA MASANORI [US]; LIU WENHAI [US]) 21 March 2013 (2013-03-21) cited in the application sequence 1 -----	39
X	US 2009/010966 A1 (DAVIS CLAUDE GEOFFREY [US] ET AL) 8 January 2009 (2009-01-08) claims 1-21; sequence 1 -----	49
X	WO 2009/014650 A2 (GEN HOSPITAL CORP [US]; SEED BRIAN [US]; WOLFE JIA LIU [US]; TSAI CHIA) 29 January 2009 (2009-01-29) page 1, lines 4-6; claims 1-54; sequence 1 -----	50
Y	A. A. HIZLI ET AL: "Phosphorylation of Eukaryotic Elongation Factor 2 (eEF2) by Cyclin A-Cyclin-Dependent Kinase 2 Regulates Its Inhibition by eEF2 Kinase", MOLECULAR AND CELLULAR BIOLOGY, vol. 33, no. 3, 1 February 2013 (2013-02-01), pages 596-604, XP055212435, ISSN: 0270-7306, DOI: 10.1128/MCB.01270-12 cited in the application figure 1b -----	1-6
A	WO 03/087348 A1 (UNIV JOHNS HOPKINS [US]; RODRIGUEZ RONALD [US]; CHOWDHURY WASIM HAIDER) 23 October 2003 (2003-10-23) claims 28,29 -----	1-78
A	IVANKOVIC M ET AL: "Site-specific mutagenesis of the histidine precursor of diphthamide in the human elongation factor-2 gene confers resistance to diphtheria toxin", MUTATION RESEARCH. GENETIC TOXICOLOGY AND ENVIRONMENTAL MUTAGENESIS, ELSEVIER, AMSTERDAM, NL, vol. 609, no. 1, 10 October 2006 (2006-10-10), pages 34-42, XP028035092, ISSN: 1383-5718, DOI: 10.1016/J.MRGENTOX.2006.06.027 [retrieved on 2006-10-10] abstract ----- -/--	1-78

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/056028

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	B. T. FOLEY ET AL: "Mutations in the Elongation Factor 2 Gene Which Confer Resistance to Diphtheria Toxin and Pseudomonas Exotoxin A: GENETIC AND BIOCHEMICAL ANALYSES", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 39, 29 September 1995 (1995-09-29), pages 23218-23225, XP055212233, ISSN: 0021-9258, DOI: 10.1074/jbc.270.39.23218 abstract; figure 2 -----	1-78

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/056028

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-78

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-78

Methods for assessing resistance or non-resistance of patients to NAD(+)-diphthamide ADP-ribosyltransferase based on presence or absence of serine phosphorylation of eEF2 protein, methods of treatment of said patient based on said concept.

2. claims: 79-102

Monoclonal antibody selectively binding to eEF2 protein that is phosphorylated at the S595 residue and its use in in vitro methods of assessing resistance /non-resistance to NAD(+)-diphthamide ADP-ribosyltransferase treatment and its use as medicament in said responsive/non-responsive therapeutic treatments.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/056028

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