NOVEL ANTIBODY MOLECULES AND NUCLEIC ACIDS BINDING TO FUNGAL STRESS PROTEIN HSP90

The present invention relates to novel antibody molecules specifically binding to fungal stress protein hsp90, nucleic acids encoding such peptides and pharmaceutical compositions and uses thereof.

An antibody fragment binding to the hsp90 fungal stress protein as well as therapeutic uses of it has been described e.g. in WO01/76627 or WO05/102386. The antibody fragment, also known as Mycograb® (Efungumab), is a fusion protein comprising the V_H and V_L domains of immunoglobulin connected by a linker peptide. Such antibody fragments are also known as "single chain variable fragment" (scFv). Mycograb® is produced by fermentation in *E.coli* in the form of inclusion bodies, which are extracted from the cell mass, refolded and subsequently purified by chromatographic steps under denaturing conditions. Characterization studies performed under native conditions have indicated that the efungumab protein has a tendency to form multimers or aggregates (the terms "multimers" and "aggregates" are used interchangeably herein). Such aggregates, in particular high molecular weight aggregates, may not be desirable for therapeutic uses. Thus, for therapeutic uses it may be desirable to eliminate or reduce the high molecular weight aggregates or to control aggregation such that the number of momomers, which a majority of such aggregates contain, are in a certain range, e.g. between 10 and 100 monomers, such as e.g. between 11 and 73 or 26 and 57 monomers.

The present invention now provides improved scFv peptides binding to hsp90 fungal stress protein, which have advantageous properties with respect to e.g. folding properties and/or formation of aggregates. The peptides of the invention are thus particularly useful for therapeutic uses.

According to one aspect of the present invention, there is provided a scFv peptide comprising a V_H domain and a V_L domain linked by an amino acid spacer, wherein the V_H domain comprises a sequence with at least 80% sequence identity to the sequence of

SEQ ID NO. 64 and the V_L domain comprises a sequence with at least 80% sequence identity to the sequence of SEQ ID NO. 66 and wherein the scFv peptide comprises an additional feature selected from the group consisting of:

(a) a substitution or deletion of an amino acid in the V_H domain at a position corresponding to that selected from the group consisting of: C_{28}, I_{29}, H_{68}, N_{85}, C_{97} and combinations thereof;

(b) a substitution or deletion of an amino acid in the V_L domain at a position corresponding to that selected from the group consisting of: V_2, V_3, F_{10}, F_{14}, A_{39}, N_{76} and combinations thereof;

(c) the amino acid spacer comprises the sequence $(GGGGS)_n$ wherein n is between 4 and 6;

(d) the V_H domain further comprises an N-terminal pelB signal sequence comprising the sequence of SEQ ID NO. 68 or a sequence having at least 80% sequence identity thereto;

(e) the V_L domain is located at the N-terminal end of the V_H domain; and

(f) combinations of features (a) to (e).

It is preferred that the V_H domain comprises a sequence with at least 90%, 95%, 99% or 100% identity to the sequence of SEQ. ID NO. 64. It is also preferred that the V_L domain comprises a sequence with at least 90%, 95%, 99% or 100% identity of the sequence of SEQ. ID NO. 66. It is to be undertood that the additional feature is present irrespective of the level of sequence identity. For example, if the additional feature is a substitution of the amino acid at position C_{28} then this substitution is present even in embodiments where the V_H domain comprises a sequence with only 80% sequence identity to SEQ. ID NO. 64.

Conveniently, the substitution of the amino acid in the V_H domain is selected from the group consisting of: $C_{28}Y$, $C_{28}S$, $I_{29}S$, $H_{68}R$, $N_{85}S$, $C_{97}Y$, $C_{97}S$ and combinations thereof. The substitution $C_{28}Y$ is particularly preferred.

Advantageously, the substitution of the amino acid in the V_L domain is selected from the group consisting of: V_2I, V_3Q, $F_{10}S$, $F_{14}S$, $A_{39}K$, $N_{76}S$ and combinations thereof.

Preferably, the scFv peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO. 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60 and 62 wherein Xaa denotes an amino acid residue other than cysteine and wherein the N-terminal methionine residue may optionally be cleaved off. It is preferred that Xaa denotes a tyrosine residue.

In one embodiment Xaa is Tyr (Y). In another embodiment Xaa is Ala (A), Leu (L), Ile (I), Val (V), Pro (P) or Met (M); in yet another embodiment Xaa is Phe (F) or Try (W); in yet another embodiment Xaa is Gly (G); in yet another embodiment X is Ser (S) or Thr (T); in yet another embodiment Xaa is Glu (E) or Asp (D); in yet another embodiment Xaa is Gln (Q) or Asn (N); in yet another embodiment Xaa is Arg (R), Lys (K) or His (H).

Preferably the scFv peptide further comprises a purification tag, more preferably a sequence of 6 histidine residues at the C-terminus.

In accordance with another embodiment of the present invention, there is provided a scFv peptide consisting of, or consisting essentially of, an amino acid sequence as set forth SEQ ID NO. 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60 and 62 wherein said peptides may optionally comprise a purification tag such as e.g. a His-Tag (e.g. as set forth in SEQ ID NO. 10, 22 or 34).

The purification Tags typically do not contribute to the therapeutic effect of the molecule and may therefore be removed after purification of the scFv fragments of the present invention.

In accordance with another aspect of the present invention, there is provided a scFv peptide comprising an amino acid sequence as set forth in SEQ ID NO. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60 and 62. In one embodiment, there is provided a scFv peptide consisting of, or consisting essentially of, an amino acid sequence as set forth SEQ ID NO. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56,

58, 60 and 62 wherein said peptides may optionally also comprise a purification tag such as e.g. a His-Tag (e.g. as set forth in SEQ ID NO. 2, 4 or 20).

As readily appreciated by the skilled person, the first Met residue of the peptides of the present invention may be also cleaved off in vivo, e.g. by *E. coli* MAP (methionine amino peptidase) if expressed in *E. coli*.

The scFv peptides of the present invention comprise two domains linked by an amino acid spacer (the terms "spacer" and "linker" are used interchangeably), e.g. having the amino acid sequence $(GGGGS)_n$ wherein n is an integer from 1 to 12, e.g. 1, 2, 3, 4 or 5. One of the domains, designated as V_H, corresponds to the heavy chain part of the antibody fragment (corresponding e.g. to amino acid residues 2 to 122 in the scFv fragment of amino acid sequence set forth in SEQ ID NO. 2 and SEQ ID NO. 30, or amino acid residues 132 to 152 in SEQ ID NO. 32). The other domain, designated as V_L, corresponds to the light chain part of the antibody fragment (corresponding e.g. to amino acid residues 138 to 246 in SEQ ID NO. 2, or amino acid residues 138-246 in SEQ ID NO. 12, or amino acid residues 2 to 110 in SEQ ID NO. 32). The VH or the VL domain may be located at the N-terminus of the scFv peptides of the present invention, i.e. the molecules may be linked as follows: VH-linker-VL or VL-linker-VH.

The optional pelB signal sequence results in subcellular localisation of the peptide to the periplasmic membrane, when expressed in E. coli, in order to improve solubility of the peptide.

In one embodiment, there is provided a scFv fragment comprising an amino acid sequence as set forth in SEQ ID NO. 30 or 32. In another embodiment, there is provided a scFv peptide consisting of, or consisting essentially of, an amino acid sequence as set forth SEQ ID NO. 30 or 32 wherein said peptides may optionally also comprise a purification tag such as e.g. a His-Tag.

In one aspect, the present invention provides scFv fragments comprising a VH and VL domain and a linker according to the present invention (e.g. as set forth in SEQ ID NO. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 30, 32 or 34) having at least one amino acid

substituted at one or more of the following positions: C29X, I30X, H69X, N86X, C98X, V139X, V140X, F147X, F151X, A176X, N213X, wherein X denotes an amino acid other than as set forth in SEQ ID NO. 2 (the numbering is as set forth in SEQ ID NO. 2 and corresponding amino acid positions in other mutants can be easily determined). In a preferred embodiment, the present invention provides scFv fragments having at least one of the following amino acid substitution: C29Y or C29S, I30S, H69R, N86S, C98Y or C98S, V139I, V140Q, F147S, F151S, A176K, N213S. It is to be appreciated that numbering of amino acids in relation to this aspect includes the N-terminal methionine residue.

In one embodiment, there is provided a scFv fragment comprising an amino acid sequence as set forth in SEQ ID NO. 24, 26 or 28. In another embodiment, there is provided a scFv peptide consisting of, or consisting essentially of, an amino acid sequence as set forth SEQ ID NO. 24, 26 or 28 wherein said peptides may optionally also comprise a purification tag such as e.g. a His-Tag.

The peptides of the present invention are useful as therapeutics. Accordingly, in one aspect of the present invention, there is provided a pharmaceutical composition comprising a scFv peptide according to the present invention, e.g. comprising an amino acid sequence as set forth in SEQ ID NO. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60 and 62 wherein Xaa is defined as above, in combination with a pharmaceutically acceptable excipient, diluent or carrier. Details of suitable excipients are provided in Remington's Pharmaceutical Sciences and US Pharmacopoeia, 1984, Mack Publishing Company, Easton, PA, USA. Exemplary excipients include pharmaceutical grade (Ph Eur) Urea and L-Arginine (Ph Eur). For example, a typical formulation of an scFv peptide of the invention is 10mg of pure scFv peptide, 150mg of pharmaceutical grade (Ph Eur) Urea and 174mg L-Arginine (Ph Eur) reconstituted in 5ml water.

An scFv peptide or a pharmaceutical composition of the invention may be administered in a dosage in the range of 0.1 to 10 mg/kg body weight of the patient. A dosage in the range 0.5 to 5 mg/kg body weight is preferred, with a dosage of around 1mg/kg being particularly preferred. The pharmaceutical composition may be administered orally.

The peptides of the present invention are useful in the treatment of fungal infections e.g. as disclosed in WO01/76627 or WO05/102386 each of which is hereby incorporated by reference. For example, the peptides of the present invention are useful in the treatment of systemic fungal infections such as invasive candidiasis or invasive aspergillosis or invasive meningitis e.g. virulent Candida species C. albicans, C. tropicalis and C. krusei and the less virulent species C. parapsilosis and Torulopsis glabrata. The peptides of the present invention are also useful in the treatment of infections by Candida, Cryptococcus, Histoplasma, Aspergillus, Torulopsis, Mucormycosis, Blastomycosis, Coccidioidomycosis, Paracoccidioidomycosis organism or malaria. Accordingly, the present invention provides a method of treating a patient with a fungal infection comprising administering to the patient an effective amount of a scFv peptide of the present invention, e.g. comprising an amino acid sequence as set forth in SEQ ID NO. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60 and 62 wherein Xaa is defined as above. The N-terminal Met may optionally be cleaved off.

The peptides of the present invention are particularly useful for combination therapies. Accordingly, in another aspect, the present invention provides a composition or a combined preparation comprising a scFv peptide of the present invention, e.g. comprising an amino acid sequence as set forth in SEQ ID NO. 2, 4, 6, 8, 10, 12, 14, 16, 18 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60 and 62 (the N-terminal Met may optionally be cleaved off), wherein Xaa is defined as above, and a antifungal agent such as e.g. a polyene antifungal or a echinocandin antifungal or an azole antifungal. Examples of antifungals useful as combination partners of scFv peptides of the present invention include e.g. amphotericin B, derivatives of amphotericin B such as AmBisome, amphotericin-B lipid complex (Abelcet), amphotericin-B colloidal dispersion (Amphocil) and amphotericin-B intralipid emulsion; nystatin; 5-fluorocytosine; caspofungin, anidulafungin, micafungin, LY303366; azoles such as isavuconazole, voriconazole, itraconazole, fluconazole, miconazole, ketoconazole, posaconazole, anidulafungin, micafungin, griseofulvin, terbinafine. Though such combination may be a fixed dose combination, generally, the scFv peptide and its combination partner are not packaged as fixed dose combinations.

The combined preparations of the present invention may be for simultaneous, separate or sequential use in the treatment of fungal infections. The peptides of the present invention may also be used in combination with more than one antifungal agent, e.g. with amphotericin B and 5-fluorocytosine, a fungin and Amphotericin B or an echinocandin plus azole.

In another embodiment, the present invention provides a method of treating a patient with a fungal infection comprising administering to the patient an effective amount of a scFv peptide of the present invention, e.g. comprising an amino acid sequence as set forth in SEQ ID NO. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60 and 62 (the N-terminal Met may optionally be cleaved off), wherein Xaa is defined as above, and at least one of the antifungal agents described above. Preferred combination partners are amphotericin B or derivatives of amphotericin B, caspofungin, anidulafungin, micafungin, voriconazole, itraconazole. The combination partners may be administered simultaneously, separately or sequentially.

In one embodiment of the present invention, the fungus causing the infection is resistant or partially resistant against an antifungal combination partner of the peptides of the invention.

The peptides of the present invention are also useful in the treatment of cancer, or a condition involving raised levels of TNFα and/or IL-6 such as autoimmune diseases or sepsis e.g. as disclosed in WO06/003384 or WO07/077454 (PCT/GB2007/000029) each of which is hereby incorporated by reference. For instance, the peptides of the present invention are useful in the treatment of leukemia such as e.g. lymphoid (lymphocytic) leukaemia (CLL), acute myeloid (myeloblastic) leukeemia (AML), acute lymphoid (lymphoblastic) leukeemia (ALL), chronic myeloid leukaemia (CML), carcinoma of the breast, carcinoma of the colon, prostate, multiple myeloma; or for the treatment of sepsis targeting human hsp90 (WO07/077454). Accordingly, the present invention provides a method of treating a patient with a cancer disease or a condition inviolving raised levels of TNFα and/or IL-6 (e.g. autoimmune disease, SIRS or sepsis) comprising administering to the patient an effective amount of a scFv peptide of the present

invention, e.g. comprising an amino acid sequence as set forth in SEQ ID NO. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60 and 62 (the N-terminal Met may optionally be cleaved off), wherein Xaa is defined as above.

In some embodiments, the autoimmune disease is Crohn's disease, rheumatoid arthritis, ulcerative colitis or systemic lupus erythermatosus.

The peptides of the present invention are useful for combination therapies with anticancer agents. Examples of suitable anticancer agents include doxorubicin, daunorubicin, epirubicin, herceptin, docetaxel, cisplatin, imatinib (Gleevec®), paclitaxel, cytarabine or hydroxyurea. Accordingly, the present invention provides a composition or a combined preparation comprising a scFv peptide of the present invention, e.g. comprising an amino acid sequence as set forth in SEQ ID NO. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60 and 62 (the N-terminal Met may optionally be cleaved off), wherein Xaa is defined as above, and a anticancer agent selected from the group consisting of doxorubicin, daunorubicin, epirubicin, herceptin, docetaxel, cisplatin, imatinib, paclitaxel and hydroxyurea. Also provided are methods of treating a patient with a cancer disease comprising administering to the patient in need an effective amount of a scFv of the present invention, e.g. peptide comprising an amino acid sequence as set forth in SEQ ID NO. 2, 4, 6, 8, 10, 12, 14, 16, 18 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60 and 62 (the N-terminal Met may optionally be cleaved off), wherein Xaa is defined as above, and at least one of the anticancer agent selected from the group consisting of doxorubicin, daunorubicin, epirubicin, herceptin, docetaxel, cisplatin, imatinib, paclitaxel and hydroxyurea.

In accordance with another aspect of the present invention, there are provided improved nucleic acid molecules encoding scFv peptides as described and improved nucleic acid constructs which are particularly useful for expressing such scFv peptides e.g. in *E. coli*. The nucleic acid constructs of the present invention for instance lead to improved expression of the scFv peptides in *E. coli*, e.g. with respect to homogeneity and titer of the expressed scFv peptide.

Preferably, the nucleic acid molecule further comprises the sequence $(taa)_n$ located at the 3' end of the sequence encoding the scFv peptide wherein n is 1 or 2.

According to another aspect of the present invention, there is provided a nucleic acid molecule comprising a sequence encoding a V_H domain comprising a sequence having at least 80% sequence identity to the sequence of SEQ ID NO. 64 and a V_L domain comprising a sequence having at least 80% sequence identity to the sequence of SEQ ID NO. 66 and further comprising the sequence $(taa)_n$ located at the 3' end of the sequence encoding the V_H or V_L domains wherein n is 1 or 2. The provision of multiple stop codons at the 3' terminus avoids erroneous read-through events.

In another aspect the present invention provides a nucleic acid molecule, e.g. a DNA or RNA molecule, comprising a nucleotide sequence as set forth in SEQ ID NO. 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, or 61 wherein nnn denotes a codon coding for an amino acid other than Cys. For instance, in one embodiment, nnn may code for Tyr such as e.g. TAT. In another aspect, the present invention provides a nucleic acid molecule comprising a nucleotide sequence as set forth in SEQ ID NO.1, 3, 5, 11, 15 or 19. As appreciated by the skilled person, nucleic acid sequences can be readily modified without altering the encoded amino acid sequence. Nucleic acid molecules based on a nucleotide sequence comprising a nucleotide sequence as set forth in SEQ ID NO. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, or 61. with one or more (e.g. up to 10, 20, 50 or 100) such silent mutations are also comprised within the scope of the present invention. Further encompassed are nucleic acid molecules which have (i) at least 80% identity, preferably at least 90%, 95%, 99% or 100% identity to SEQ. ID NO. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, or 61; or (ii) hybridize under high stringency conditions to the nucleic acid molecules having a sequence as set forth in SEQ ID NO. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, or 61. The term high stringency conditions is readily understood by the skilled person and may refer, e.g., to washing in 6X SSC/0.05% sodium pyrophosphate at 37°C. (for 14-base oligos), 48°C. (for 17-base oligos), 55°C.

(for 20-base oligos), and 60°C. (for 23-base oligos). Suitable ranges of such stringency conditions for nucleic acids of varying compositions are described in Krause and Aaronson (1991) Methods in Enzymology, 200:546-556.

In one embodiment, the present invention provides a vector molecule comprising a nucleotide sequence of a nucleic acid molecule of the invention, e.g. as set forth in SEQ ID NO. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, or 61. Preferably, such vector molecule is suitable for expressing the nucleic acid molecules as set forth in SEQ ID NO. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, or 61. in e.g. E. coli. Suitable expression vectors are readily known to the skilled person. An example of suitable vector includes for instance pGEX or pET. Another embodiment provides a host cell, e.g. E. coli, comprising such a vector molecule.

In another embodiment there is provided a method for producing a scFv peptide of the present invention, e.g. comprising an amino acid sequence as set forth in SEQ ID NO. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60 or 62 (the N-terminal Met may optionally be cleaved off), wherein Xaa is defined as above which comprises culturing a host cell having incorporated therein an expression vector containing under control of suitable transcriptional control elements a nucleic acid sequence of a nucleic acid molecule of the invention e.g. as described in SEQ ID NO. 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, or 61 under conditions sufficient for expression of said peptides in the host cell, e.g. *E. coli*, thereby causing the production of said peptide; and recovering the peptide produced by said cell.

The percentage "identity" between two sequences is determined using the BLASTP algorithm version 2.2.2 (Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-PLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402) using default parameters. In particular, the BLAST algorithm can be accessed on the Internet using the URL http://www.ncbi.nlm.nih.gov/blast/.

Brief Description of the Figures

Figure 1 is a diagram showing schematically the sequence of the wild type Mycograb scFv peptide and Mycograb mutants. Stop codons of the nucleic acid molecules encoding the respective peptides are also shown at the C-terminal end.

Figure 2 is a diagram showing the principle of the ELISA assay of Example 2.

Figure 3 is a graph in which the black bars show yield after solubilization with NLS of all investigated mutants. The error bars show the Standard deviation for samples analyzed twice. The white bars are a graphic representation of mass balance after NLS refolds of all investigated mutants. Mutants are ranked according to increasing refolding recovery values.

Figure 4 shows graphs indicating the recovery after refolding for 5 selected mutants when urea and DTT was used as solubilizing agent (A) and when GuHCl and DTT were used as solubilizing agent (B). White bars: Recovery when mass of protein found in the IB.SOL solution was used for calculation (equ. 1) Black bars: Refolding recovery when mass of protein found in the IB.RES solution is used for calculation.

Figure 5 is a graph showing the time required for a visible beginning clarification of a solubilization solution after addition of 4% NLS (white bars) and the time required until no further clarification was observed (black bars) for all tested mutants. Mutants are ranked according to the start time in ascending order.

Figure 6 is a variability chart for the response start of solubilization and indicates number of cysteines, number of linker elements and if the heavy (vh) or light chain (vl) fragment was at the N-terminus. 1: Mutant Myc 106 had the fastest solubilization start but contained 5 cysteines.

Figure 7 shows chromatograms as an overlay of REF end samples of Mutants MYC 135, Myc 130, Myc 133, Myc 119, Myc 123 wild type and Myc 116.

Figure 8 shows chromatograms as an overlay of REF end samples of Mutants MYC 134, Myc 137, Myc 138, Myc 106 Myc 136, Myc 123 and Myc 139.

Figure 9 shows scaled estimates and a prediction profiler of the following parameters: linker length, number of cysteines and Vh/Vl arrangement for the response retention time of mutants. The scaled estimates predict to what extent the retention time would shift when the parameter is increased from centerpoint (the red number in the prediction profiler plot on the x-axis) to a higher level.

Figure 10 is a plot of linker length versus retention time measured in RP-HPLC (RPC2) for tested mutants. The early retention time of MYC 130 compared with the other mutants is highlighted.

Figure 11 shows a normalized overlay of all REF.END samples from Figures 7 and 8 for estimation of peak area from peak 2.

Figure 12 shows a RP-HPLC 2 chromatogram overlay of a REF.End sample of MYC 119 solubilized with 8M urea +DTT, 8M urea, 6M GuHCl +DTT and 6M GuHCl dilution was 1:50 with a buffer containing 20mM Tris, 2mM cysteine, 1% NLS, pH 9.0.

Figure 13 is an RPC 2 chromatogram of a REF.End sample of MYC 119 after solubilization with 6M urea and 5mM DTT and subsequent refolding by a 1:10 dilution.

Figure 14 is an image of a gel following SDS Page analysis of REF.IM and REF.END sample of MYC 119 after solubilization with 6M urea and refolding by a 1:10 and 1:50 dilution, respectively. Lanes 1-8: non reducing SDS Page, lanes 9-14: reducing SDS Page. R= reducing; n-r = non reducing

Figure 15 is an RP-HPLC chromatogram overlay (RPC 2) of an inclusion body sample from mutant MYC 119 after solubilization with 6 M urea (black) and 4 % NLS (blue).

Figure 16 shows images of: left gel: Reducing (r) SDS-Page for Mutants MYC 118, 119, 130, 133, 134, 135 and 137; and right gel: Non-reducing (n-r) SDS Page of the same samples

Figure 17shows images of:: left gel: Reducing SDS-Page for Mutants MYC 106, 123 wt, 136, 138, 139 and 140; and right gel: Non-reducing SDS Page of the same samples

Figure 18 is an overlay of SEC HPLC 0.5% NLS chromatograms of REF.End samples for the mutants Myc 118, Myc 119, Myc 130, Myc 133 and Myc 135. IBs from these mutants were isolated at bench scale.

Figure 19 is an overlay of SEC HPLC 0.5% NLS chromatograms of REF.End samples for the mutants Myc 134, Myc 136, Myc 137, Myc 138, Myc 139, Myc 140, Myc 106 and Myc 123 wild type. IBs from these mutants were isolated in the pilot plant.

Figure 20 shows a scatter plot and linear regression (continuous line) of measured MW versus theoretical MW of REF.End samples for all mutants. The 95% confidence interval for the fit is also shown (dashed line). The dot at top left shows MYC 130. The dots within the dashed lines are within the 95% CI and therefore not significantly different from the wildtype. The dots below both dashed lines represent mutants with lower average MW than predicted and the dots above both dashed lines represent mutants where a higher average MW was measured than predicted.

Figure 21 shows SEC-HPLC (formulation) chromatograms for REF.END samples of Myc 119, Myc 106-origami, Myc 123 wt and Myc 137 after UFDF against 50mM Tris, pH 9.0 buffer. Samples were taken after each volume reconstitution. Sample prior to UFDF treatment (5), after 1st 'buffer exchange step (2), 2nd buffer exchange step (3), 3rd (4) and last (5) step.

Figure 22 shows RP-HPLC 2 chromatograms of REF.IM, REF.3T and REF. END samples for all tested mutants:

Brief Description of the Sequence Listings

SEQ ID NO. 1 is Myc123

SEQ ID NO. 2 is the peptide sequence encoded by SEQ ID NO. 1

SEQ ID NO. 3 is Myc102, Mycograb-6H-TAA

SEQ ID NO. 4 is the peptide sequence encoded by SEQ ID NO. 3

SEQ ID NO. 5 is Myc101, Mycograb-TAA

SEQ ID NO. 6 is the peptide sequence encoded by SEQ ID NO. 5

SEQ ID NO. 7 is MycC29X-TAA, e.g.: Myc105, MycC29Y-TAA

SEQ ID NO. 8 is the peptide sequence encoded by SEQ ID NO. 7

SEQ ID NO. 9 is MycC29X-6H-TAA, e.g.: Myc106, MycC29Y-6H-TAA, Myc113, MycoC29S-6H-TAA

SEQ ID NO. 10 is the peptide sequence encoded by SEQ ID NO. 9

SEQ ID NO. 11 is Myc107, Myco-4-TAA

SEQ ID NO. 12 is the peptide sequence encoded by SEQ ID NO. 11

SEQ ID NO. 13 is MycoC29X-4-TAA, e.g.: Myc108, MycoC29Y-4-TAA; Myc114, MycoC29S-4-TAA

SEQ ID NO. 14 is the peptide sequence encoded by SEQ ID NO. 13

SEQ ID NO. 15 is Myc109, N-Myco-4-TAA

SEQ ID NO. 16 is the peptide sequence encoded by SEQ ID NO. 15

SEQ ID NO. 17 is N-MycoC29X-4-TAA, e.g.: Myc110, N-MycoC29Y-4-TAA

SEQ ID NO. 18 is the peptide sequence encoded by SEQ ID NO. 17

SEQ ID NO. 19 is Myc111, N-Myco-6H-TAA

SEQ ID NO. 20 is the peptide sequence encoded by SEQ ID NO. 19

SEQ ID NO. 21 is N-MycoC29X-6H-TAA, e.g.: Myc112, N-MycoC29Y-6H-TAA

SEQ ID NO. 22 is the peptide sequence encoded by SEQ ID NO. 21

SEQ ID NO. 23 is Myc115, MycYSSS

SEQ ID NO. 24 is the peptide sequence encoded by SEQ ID NO. 23

SEQ ID NO. 25 is Myc116, MycYSIQSS

SEQ ID NO. 26 is the peptide sequence encoded by SEQ ID NO. 25

SEQ ID NO. 27 is Myc117, MycSIQKS

SEQ ID NO. 28 is the peptide sequence encoded by SEQ ID NO. 27

SEQ ID NO. 29 is Myc118, VH-2Bam-2VL

SEQ ID NO. 30 is the peptide sequence encoded by SEQ ID NO. 29

SEQ ID NO. 31 is Myc119, VL-2Bam-2VH

SEQ ID NO. 32 is the peptide sequence encoded by SEQ ID NO. 31

SEQ ID NO. 33 is Myc145, MycC98X-6H-TAA

SEQ ID NO. 34 is the peptide sequence encoded by SEQ ID NO. 33

SEQ ID NO. 35 is Myc129 (MycYSRIQSS)

SEQ ID NO. 36 is the peptide sequence encoded by SEQ ID NO. 35

SEQ ID NO. 37 is Myc130 (MycYSRSIQSSKS)

SEQ ID NO. 38 is the peptide sequence encoded by SEQ ID NO. 37

SEQ ID NO. 39 is Myc133

SEQ ID NO. 40 is the peptide sequence encoded by SEQ ID NO. 39

SEQ ID NO. 41 is Myc134

SEQ ID NO. 42 is the peptide sequence encoded by SEQ ID NO. 41

SEQ ID NO. 43 is Myc135

SEQ ID NO. 44 is the peptide sequence encoded by SEQ ID NO. 43

SEQ ID NO. 45 is Myc136

SEQ ID NO. 46 is the peptide sequence encoded by SEQ ID NO. 45

SEQ ID NO. 47 is Myc137

SEQ ID NO. 48 is the peptide sequence encoded by SEQ ID NO. 47

SEQ ID NO. 49 is Myc138

SEQ ID NO. 50 is the peptide sequence encoded by SEQ ID NO. 49

SEQ ID NO. 51 is Myc139

SEQ ID NO. 52 is the peptide sequence encoded by SEQ ID NO. 51

SEQ ID NO. 53 is Myc140

SEQ ID NO. 54 is the peptide sequence encoded by SEQ ID NO. 53

SEQ ID NO. 55 is Myc141

SEQ ID NO. 56 is the peptide sequence encoded by SEQ ID NO. 55

SEQ ID NO. 57 is Myc142

SEQ ID NO. 58 is the peptide sequence encoded by SEQ ID NO. 57

SEQ ID NO. 59 is Myc143

SEQ ID NO. 60 is the peptide sequence encoded by SEQ ID NO. 59

SEQ ID NO. 61 is Myc144

SEQ ID NO. 62 is the peptide sequence encoded by SEQ ID NO. 61

SEQ ID NO. 63 is the nucleotide sequence encoding the heavy chain of the wild type Myc123 scFv peptide.

SEQ ID NO. 64 is the peptide sequence encoded by SEQ ID NO. 63

SEQ ID NO. 65 is the nucleotide sequence encoding the light chain of the wild type Myc123 scFv peptide.

SEQ ID NO. 66 is the peptide sequence encoded by SEQ ID NO. 65.

SEQ ID NO. 67 is the nucleotide sequence of the pelB signal sequence.

SEQ ID NO. 68 is the peptide sequence encoded by SEQ ID NO. 67.

SEQ ID NO. 69 is the epitope from Candidal hsp90 for which the scFv peptide of SEQ ID NO. 2 (Mycograb) is specific.

SEQ ID NO. 70 is the epitope of a scrambled peptide used in the binding assay of Example 2.

Experimental

Example 1

E. coli host cells are transformed with the expression vector and cultivated in submers culture. At suitable OD600, expression of scFv is induced by derepression or activation of the inducible promorter (i.e. tac, trc or T7-lac promoter). This induction leads to accumulation of scFv in the host cell, resulting in production of insoluble inclusion bodies mainly made of aggregated scFv. After a suitable expression period, cells are harvested by centrifugation and disrupted. The insoluble inclusion bodies are subsequently isolated by gravimetric means.

The DNA sequences set forth in SEQ ID NO. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59 or 61 are inserted into an expression vector suitable for E. coli (i.e. pET). The protein is expressed in a Escherichia coli host and then purified by affinity chromatography. Standard molecular biology protocols are employed (see, for example, Harlow & Lane, supra; Sambrook, J. et al., 1989, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Sambrook, J. & Russell, D., 200 1,

Molecular Cloning: A Laboratory Manual, 3rd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor).

After intracellular expression the scFv peptides are accumulated in the form of inclusion bodies within the E. coli cells. For purification, inclusion bodies are isolated and the product is extracted by solubilization and refolding. Purification to over 95% purity is achieved by ion exchange chromatography and immobilized metal affinity chromatography (IMAC).

Example 2 – ELISA Activity Assay

1. Summary

The binding activity of MYC123 ("wild type" Mycograb) and mutant Mycograb peptides was detected in an ELISA using the peptide epitope of hsp 90 as antigen. Mycograb or mutant Mycograb became bound to biotinylated peptide, which in turn was bound to streptavidin-coated microtitre plates. A scrambled peptide was used as a control sequence. Detection was accomplished using a peroxidase conjugated anti-His antibody, which binds to the His region of the MYC123 protein. The peroxidase reacted with the ABTS substrate to produce a green substance, the absorption of which was measured at 405 nm. The absorption at 405 nm is proportional to the activity of MYC123 in the solution. The activity was determined from the 6-point calibration curve for a reference standard and was indicated as % activity compared with the reference.

The principle of the ELISA is depicted in Figure 2 in which Streptavidin 1 is coated on a plate and is bound to biotin 2. The biotin 2 is, in turn, bound to the Hsp 90 peptide 3 which is located in the Hsp90 binding site 4 of the MYC123 scFv peptide 5. The scFv peptide 5 has a His tag 6 to which becomes bound the Anti-His-Peroxidase detection antibody 7.

2. Principle and Source of Procedure

The ELISA utilised was a direct detection assay where Mycograb or mutant Mycograb was captured using a streptavidin surface microplate coated with a biotinylated antigenic peptide (Biotin-NKILKVIRKNIVKK – epitope sequence from candidal

Hsp90). The presence of Mycograb or mutant Mycograb was then detected using an anti-His tag antibody conjugated to horse radish peroxidase. ABTS, a substrate for the horse radish peroxidise, was then added to the wells, and the concentration of Mycograb present was proportional to the absorbance measured at 405nm. The activity of samples of Mycograb or mutant Mycograb was determined directly from a standard curve generated using the pre-existing Mycograb drug product reference material.

3. Materials

3.1 Equipment

Streptawell High Bind microtitre plates were supplied by Roche (Cat No.11989685001). Assays were performed using a Bio-Rad Model 680 microplate reader. Hardware control was performed using the Microplate Manager Software version 5.2.1 (Bio-Rad, USA). Data analysis was performed using Microsoft Excel.

3.2 Chemicals and Reagents

3.2.1 Chemicals

All chemicals were of analytical grade unless otherwise stated.

Tris.....	Sigma T6791
Bovine Serum Albumin.....	Sigma A7030
Concentrated Hydrochloric acid.....	Sigma 32033-1
Phosphate Buffered Saline Tablets....	Sigma P4417
Tween 20.....	SigmaUltra P7949
Anti-His tag antibody HRP conjugate	Sigma A7958
ABTS.....	Sigma A3219
Biotin- NKILKVIRKNIVKK (SEQ. ID NO: 69)	Pepceuticals, UK
Biotin- SFKWGVTTSLSYFPK (SEQ. ID NO: 70)	Pepceuticals, UK

Water, Milli-Q water 18.2MΩfiltered 0.22µm pore size.

3.2.2 Reagents

Blocking buffer Stock 1 (5% w/v BSA in Milli-Q water)

BSA.....2.5g

Weighed out 2.5g of BSA and added to 50mL of Milli-Q water. Store at 4°C for 1 week.

1M Tris buffer pH 7.8

Tris.....121.24g

Weighed out 121.24g Tris and dissolved in 950ml Milli-Q water with stirring. Checked and adjusted pH with drop-wise addition of Concentrated Hydrochloric Acid until pH was 7.8. Made up to 1litre with Milli-Q water. Filtered through a 0.22µm filter (Sartorius) and stored at room temperature for up to 1 month.

Sample Diluting Buffer (20mM Tris pH 7.8 0.1% w/v BSA)

1mL of 1M Tris stock solution was added to 48ml of Milli-Q water and 1ml of Blocking buffer Stock 1 solution. Made fresh for each experiment.

Wash Buffer (PBS + Tween 20 0.1% v/v)

Dissolved 5 PBS tablets in 900mL of Milli-Q water, added 1mL of Tween 20, stirred until tablets had dissolved and made up to 1000mL with Milli-Q water. Stored at 4°C for up to 1 week.

Blocking buffer Stock 2 (Wash Buffer + 5% w/v BSA)

BSA.....2.5g

Weighed out 2.5g of BSA and dissolved in 50mL of Wash Buffer. Stored at 4°C for 1 week.

Peptide Diluting Buffer (PBS + 0.1% v/v Tween 20 + 0.1% w/v BSA)

1mL of Blocking buffer Stock 2 was added to 49mL of Wash buffer. Made fresh for each experiment.

Antigenic peptide solution

Biotin-NKILKVIRKNIVKK peptide.....10mg.

A 2mg/ml solution of the custom synthesised antigenic peptide solution was made up by weighing out 10mg of peptide and dissolving it in 5ml of Milli-Q water. 50-100µl

aliquots were dispensed into 1.5ml Eppendorf tubes and stored frozen at -80°C for up to one year.

Antigenic peptide Working Solution (4µg/mL peptide in Peptide Diluting Buffer)

25µL of Antigenic peptide solution (2mg/mL) was added to 12.475mL of Peptide Diluting Buffer to give a 4µg/mL solution. Made fresh for each experiment.

3.2.3 Sample Preparation

Control Article

Resuspended 1 x 10mg Mycograb reference batch (BN270603) in 5ml of Milli-Q water, mixed gently to ensure all the powder in the vial was incorporated and dissolved. Centrifuge at 13,000rpm for 5 minutes to remove any particulate matter. The protein concentration of this solution was then determined according to the standard UV protein concentration procedure.

Test Article

Resuspended 1 x 10mg Mycograb or mutant Mycograb test material in 5ml of Milli-Q water, and processed in an identical fashion to the Control Article.

Calibration curve standards

Control Article material was diluted in Sample Diluting buffer to give 5mL of a 5µg/mL top concentration sample. This solution was then used to generate two-fold serially diluted samples each in a final volume of 2mL over a concentration range of 5-0.156µg/mL.

4. Procedure

1. An aliquot of the 2mg/ml stock solution of antigenic peptide was removed from the freezer and diluted 1:500 (25µl peptide in 12.5ml buffer) with PBS buffer containing 0.1%(w/v) BSA and 0.1%(v/v) Tween 20 to generate a working solution of 4µg/ml peptide. A 96 well high bind StreptaWell plate (Roche) was coated from rows B-H with 100µl of 4µg/ml biotin-NKILKVIRKNIVKK peptide in 0.1% (w/v) BSA PBS-0.1%

(v/v) Tween 20. 100µl per well of 0.1% (w/v) BSA PBS-0.1% (v/v) Tween 20 were added to all wells in Row A. The plate was then stored overnight at 4°C.

2. The plate wells were then washed 3x 30sec with 200µl of PBS 0.1% (v/v) Tween 20 buffer on a Thermo WellWash AC.

3. Mycograb and mutant Mycograb samples were prepared prior to loading by diluting down to 5µg/ml in 20mM Tris buffer pH 7.8, 0.1% (w/v) BSA from the resuspended Mycograb vial stock solution. Mycograb® samples were then prepared from this initial 5µg/ml solution by serial dilution x2 down to 0.15625µg/ml with 20mM Tris buffer pH 7.8, 0.1% (w/v) BSA. All the individual dilutions were performed in either 1.5ml Eppendorf tubes (VWR Cat No 211-2139) or 7ml Bijoux containers (VWR Cat. No. 215-0328), depending on the amount required for the experiment.100µl of each dilution sample was then loaded onto the plate in triplicate. A control set of blank wells containing 100µl 20mM Tris pH 7.8, 0.1% (w/v) BSA was also included in Row H.

4. The plate was left at room temperature for 1 hour and the wells then washed 3 times with PBS-0.1% (v/v) Tween 20 as described in step 2.

5. 100 µl of mouse monoclonal Anti-His HRP conjugate (Sigma A7058) was loaded into each well at a concentration of 1:2000 in 0.1% (w/v) BSA PBS-0.1% (v/v) Tween 20 and left for 1 hour at room temperature.

6. Wells were then washed as described above and the bound Mycograb® detected by the addition of 100µl of ABTS® reagent. The colourimetric development was read at 405nm with readings taken when the absorbance of the highest concentration samples in the second calibration curve reached 1.3 AU. The concentration of Mycograb® was proportional to the absorption at A405nm.

7. The A405nm absorbance results for the reference material were transferred into an Excel spreadsheet and a 6-point second order calibration function $y = a + bx + cx^2$ was plotted from the reference sample A405nm minus blank versus concentration of

Mycograb® in µg/ml with a correlation coefficient of ≥ 0.99. If an observed 'hook' effect existed, the highest concentration point was removed from the graph, leaving a 5-point calibration curve. Two individual well outliers (as determined by eye) per plate was removed from the data analysis under some circumstances, provided that there is no more than one outlier per triplicate measurement. Percentage activity was calculated using non-linear regression analysis to calculate apparent concentrations in the samples using the appropriate absorbance means.

The ELISA results obtained during the study are shown in Table 1.

Table 1

Sample information				**Bioassay data**	
No.	**Sample**	**Sample info**	**Buffer composition**	**ELISA Site 1** [%]	**ELISA Site 2** [%]
1	MYC123 WT DF.R	Wild type	50 mM Tris, pH 9.0	71	95
2	DP (#140602)	Wild type	0.5 M urea, 0.2 M L-arginine pH 9.5	90	78
3	MYC123 WT DOW.FT	Wild type	50 mM Tris, 1 M sucrose, ~0.2% Tween20, 3 mM DTT, pH 3.0	135	130
4	MYC123 WT DOW.FT	Wild type	50 mM Tris, 3 mM DTT, pH 9.0	85	131
5	MYC C28Y DF.R	C28Y	50 mM Tris, pH 9.0	76	91
6	MYC C28Y DOW.FT	C28Y	50 mM Tris, 3.3 mM DTT, pH 9.0	79	98

Samples 1 and 3-6 are process intermediates (not final drug substance) obtained after prepurification of inclusion bodies, refolding and removal of detergent NLS (by Dowex chromatography or diafiltration). Sample 2 is an original wild type drug product produced by Biomeva/Thymoorgan and used in Phase III trials. The specification for the original drug product was 75 – 125% of the reference standard. All samples were judged as active (binding).

Example 3 - Minimum inhibitory concentration determination of *Cryptococcus neoformans*

Summary

In the MIC assay the antimycotic activity of MYC123, using *Cryptococcus neoformans* as model organism, was determined. This assay measures antifungal activity and may mimic the action of MYC123 in the clinical setting.

The MIC of MYC123 was determined by broth micro dilution according to the National Committee for Clinical Laboratory Standards document M27-A2 (2002). Briefly: RPMI medium was inoculated with 10^3 CFU/ ml of *C. neoformans*. MYC123 was added in decreasing concentrations to the medium (1024 µg/ml, 512 µg/ml, 256 µg/ml ...). The MIC plates were incubated at 37°C for 72h. The endpoints were determined as the concentration to produce optically clear wells (MIC-0) and the concentration resulting in a prominent decrease in turbidity (≥ 50% growth inhibition, MIC-2) compared with the growth control.

Method

Pre-Assay Preparation

Safety cabinet: A SAB plate was inoculated with *C.neoformans* and incubated for 48-72hr at 35°C. The plate was sealed with parafilm.

Bench:

RPMI was prepared. Antifungal agents were prepared according to NCCLS methodology (M27-A2) – total of 11 concentrations in RPMI growth medium. Concentrations were at 2x the final concentration required for single MIC

MIC Plate

In a U-shaped 96-well plate – 100µl of the highest drug concentration to be tested (2x required concentration) was added to well 1 of rows A and B (assay done in duplicate).

This was repeated across the columns on the plate with descending concentrations e.g. next concentration well 2 of rows A + B, next concentration well 3 of rows A + B. Well 12 contained growth medium only.

Safety Cabinet: Inoculum Preparation – Direct colony suspension. A direct colony suspension of *Cryptococcus neoformans* was made from a 48-72hr old plate into RMPI medium. This was adjusted to 0.5 MacFarlands standard (approx $1x10^6 – 5x10^6$ cfu/ml). A 1:50 dilution was made. A further 1:20 dilution (approx $1x10^3 – 5x10^3$ cfu/ml, 2x inoculum required) was made

Safety cabinet: Plate inoculation. The plate was worked from well 12 to well 1. This avoided drug carryover. 100µl of inoculum suspension was pipetted into each well (final inoculum ($0.5x10^3 – 2.5x10^3$ cfu/ml). Plates were sealed with parafilm.

Incubation

The MIC plates were incubated at 37°C for 72hrs. To check the inoculum, the inoculums suspension were serially diluted and 10µl of the dilutions were plated out onto a SAB plate and incubated at 37°C for 72hrs.

Reading Results

Using a reading mirror; growth was compared with that of the 'no-drug' control (well 12) and growth scored as follows:

0 – optically clear
1 – slightly hazy
2 – prominent decrease in growth (approx 50%)
3 – slight reduction in turbidity
4 – no reduction in turbidity

MIC results obtained during the study as shown in Table 2

Table 2

Sample information				Bioassay data
No.	**Sample**	**Sample info**	**Buffer composition**	**MIC25.02.2008/29.02.2008 [µg/ml]**
1	MYC123 WT DF.R	Wild type	50 mM Tris, pH 9.0	128
2	DP (#140602)	Wild type	0.5 M urea, 0.2 M L-arginine pH 9.5	64
3	MYC123 WT DOW.FT	Wild type	50 mM Tris, 1 M sucrose, ~0.2% Tween20, 3 mM DTT, pH 3.0	16
4	MYC123 WT DOW.FT	Wild type	50 mM Tris, 3 mM DTT, pH 9.0	64
5	MYC C28Y DF.R	C28Y	50 mM Tris, pH 9.0	64
6	MYC C28Y DOW.FT	C28Y	50 mM Tris, 3.3 mM DTT, pH 9.0	64
	Reference 070602		0.5 M urea, 0.2 M L-arginine pH 9.5	128/64
	Buffer FB		0.5 M urea, 0.2 M L-arginine pH 9.5	512 or 256
	Buffer Tris		50 mM Tris, pH 9.0	>512
	Buffer 6		50 mM Tris, 3 mM DTT, 1 M sucrose, 0.2% Tween20, pH 3.0	16

Samples 1 and 3-6 were process intermediates (not final drug substances) obtained after prepurification of inclusion bodies, refolding and removal of detergent NLS (by Dowex chromatography or diafiltration). Sample 2 was on original wild type drug product produced by Biomeva/Thymoorgan and used in Phase III trials.

The MIC results obtained for the samples were compared with the results obtained for the corresponding buffer. All the samples were regarded active in comparison to the Reference 070602 besides sample 3. Sample 3 and the Buffer 6 gave the same results demonstrating that the buffer without MYC123 was toxic for the test organism. Values

for the samples 1 and 4-6 were far from the values for Buffer Tris which indicates an increased reliability of the data.

Example 4

Summary

The aim of the Mycograb mutants was to obtain a mutant scFv peptide with improved structural properties compared with the wild type Mycograb. It was believed that through point mutations, especially the replacement of free cysteine by tyrosine, aggregation and formation of incorrect disulfide bonds during down stream processing should be reduced. It was also believed that exchanging the orientation of the heavy chain fragment with the light chain fragment and removing the HIS-Tag is be beneficial for formation of a native 3D structure of the Mycograb molecule.

After cloning, the constructs were sequenced prior to fermentation. The fermentation was scaled up to deliver enough material for inclusion body (IB) isolation and for further downstream processing. The expression constructs of the mutants were purified according to the adapted Biomeva process until the refold end step.

The physical parameters of a range of mutant Mycograb peptides was tested as set out in Examples 5 to 12.

An overview of the mutants tested and their mutations is given in Table 3. The wild type was included in the studies for comparison reasons.

Table 3: Name and structural properties of the 12 investigated Mycograb mutants and the wild type (myc 123)

MUTANT	HIS tag	Linker length	VH-VL alignment	cysteines	additional mutations
MYC118	NO	4X	VH N-terminal	5	
MYC 119	NO	4X	VL N-terminal	5	
MYC 130	NO	3X	VH N-terminal	4	C29Y,I30S, H69R, N86S, V139I, V140C F147S, F151S, A176K, N213S
MYC 133	NO	5X	VH N-terminal	5	
MYC 135	NO	5X	VL N-terminal	5	
MYC 134	NO	6X	VH N-terminal	5	
MYC 137	NO	4X	VH N-terminal	4	C29Y, I30S
MYC 138	NO	5X	VH N-terminal	4	C29Y, I30S
MYC 106 origami	YES	3X	VH N-terminal	4	oxidatives cytoplasma
MYC 136	NO	6X	VL N-terminal	5	
MYC 139	NO	6X	VH N-terminal	4	C29Y, I30S
MYC 123 Wt	YES	3X	VH N-terminal	5	
MYC 140	NO	4X	VL N-terminal	4	C29Y, I30S

The methodology used in the test assays will now be described.

Inclusion body (IB) isolation

4 L of fermentation broth obtained in LVA (Laborversuchsanstalt) from shake flask culture of Mutants Myc 118,119,130,133 and Myc 135 were disintegrated with a high pressure homogenizer (LAB 40-15 RBFI) in RPP4 at 700 Bar for 2 cycles of 15 min each. The IBs were separated from the cell debris at lab scale with a bottle centrifuge at 10 000 rpms for 20 min at 4°C. IBs were washed twice with water for laboratory use (WFL) and afterwards, a 20% (w/v) suspension in WFL was prepared. The suspension was stored in aliquots at -20°C.

IBs from mutants Myc 134,137,138,106,136,139,140 and Myc 123 (wt) were isolated at pilot scale in RPP4 because fermentation of these mutants was done at 30L scale in bioreactors. IBs were separated from cell debris with a disc stack centrifuge. A 20% suspension (w/v) in WFI was prepared. This suspension was stored in aliquots at -20°C.

Solubilization

Solubilization with NLS (according to adapted Biomeva process). Solubilization of the 20% IB suspension was done by dilution with WFL to a protein concentration of 8 mg/ml followed by a 1:2 dilution with 100mM Tris/Base, 4% NLS, pH 9.0 buffer. The solution was stirred at room temperature in a beaker until clarification but at least for 30 min. The time until start and end of clarification was recorded.

Alternative Solubilization with Urea, GuHCl, DTT. The alternative solubilization strategy was performed by a 1:10 dilution of a respective volume of 20% IB suspension with 20mM Tris 8M Urea +/- 5mM DTT or 20mM Tris, 6M GuHCl +/- 5mM DTT both at pH 9.0. The resulting concentration of urea and GuHCl was 7.2M and 5.2M respectively, due to the volume of the IB suspension solution.

Refolding of solubilized IB's

Refolding with NLS. The refold was done by 1:4 dilution of the solubilization solution with a 50mM Tris/Base buffer. The final concentration of NLS was 0.5%. Refolding was initiated by addition of 50µM $CuCl_2$. Samples were taken and immediately submitted for RPC2 analysis prior and after $CuCl_2$ addition, then approximately 24, 48, 72 and 96 hours after $CuCl_2$ addition.

Refolding after solubilization with Urea, GuHCl. Refolding of a Mycograb solution after solubilization with 8M urea or 6M GuHCl +/-DTT was performed by 1:50 dilution with a buffer containing 20mM Tris/Base, 1% NLS and 2mM Cystin at pH 9.0.

For some mutants, dilution of a Mycograb solution after solubilization with urea by 1:10 with a buffer containing 20mM Tris/Base, 0.5M L-arginine and 2mM Cystin at pH 9.0 was also performed.

The refold solution was stirred for 96 hrs at 4°C or 24hrs at RT, respectively.

Refolding kinetics

A refold kinetic was recorded for mutant Myc 119 in order to determine the required refolding time. A sample of a 20% IB suspension of Myc119 was solubilized as described above. Refolding was performed as described above, but samples were taken at respective time intervals and analyzed by RPC 2.

NLS removal by UF/DF from refolding solution. Mutants Myc119, Myc 137, Myc 106 and Myc 123 (wt) were solubilized and refolded as described above. After refolding, a buffer exchange of REF.END solution was performed with an Amicon stir cell with 10kDa molecular weight cut-off. NLS concentration after each turn over volume was determined with RP-HPLC. 50 ml of REF.END solution were concentrated to 25ml and then filled up again to 50ml with diafiltration buffer. This procedure was carried out 4 times. Aggregation tendency after NLS removal was measured with SEC-HPLC running with formulation buffer.

SDS Page analysis

SDS Page was performed using NuPAGE 4-12 BisTris gels and MOPS as running buffer. The run time was 65 minutes at 200 volt. A mass of 0.2-0.4 µg Mycograb was applied on each lane. After electrophoresis, the gels were stained with silver. For reducing SDS Page, 100 mM DTT was added to the sample.

Results

The expression construct of the mutants was analyzed at different stages in the down stream procedure with the analytical methods listed in Table 4:

Table 4. Code and description of process intermediates and the respective analytical assay

CODE	Intermediate description	Analytical method
IB.RES	Resuspension of IB's (20%w/v)	RPC 1
IB.SOL	Solubilized IB's	RPC 1, RPC 2
REF.IM	Refold intermediate prior to $CuCl_2$ addition	RPC1, RPC 2
REF.END	Refold solution, endpoint	RPC 1, RPC 2, SEC 0,5%NLS, SDS PAGE red/non red, Pep-map, denat. SEC

Table 5 gives a description of the analytical methods listed in Table 4 that were used for evaluation of the mutants. A comment is included describing the specificity of the particular assay.

Table 5: Analytical method, the respective response and Unit of Measurement (UoM) for the assays used to evaluate the mutant samples.

Analytical method	Response	UoM	Comment
RPC 1	total protein mass	mg/ml	This assay is not specific for Mycograb Sample is dissolved with SDS, DTT and urea
RPC II	chromatogram	$t_{R\ (monomer)}$ [min]	Assay can only be evaluated by overlay of chromatograms. Shift of the 'monomeric peak' indicates refolding
SEC 0,5% NLS	average molecuar weight	kDa	For samples containing 0,5% NLS. RT of peak maximum is correlated to MW. Broad elution peak from 43 -900kDa
SDS Page reducing	Monomeric/Dimeric band; Impurity content	band	Non-covalent aggregates are dissolved by reduction; in comparison to non reducing gel, semi-quantitative evaluation about aggregate content possible
SDS Page non-reducing	Monomer and aggregate bands	band	
Pep Map	Disulfide bridging	no, weak, strong and very strong signal	Ability to detect peptides depends on the sensitivity of the measuring device
SEC Formulation	average molecuar weight	kDa	For samples in a similar matrix as formulation buffer. RT of peak maximum is correlated to MW.
RPC NLS	NLS concentration	mg/ml	

Further details of the respective assays for the investigated mutants are summarized and discussed in the following Examples 5 to 12.

Example 5 - Mass balance after solubilization and refolding - results RPC1 titer determination

Protein concentration prior and after solubilization and refolding of IB's was measured with the titer assay. As this assay measures all soluble protein present in the sample, mass balance should yield 100%. Mass balance for solubilization was calculated using equ.1.

$$\% \ Sol = \frac{mg_{RPC1}\text{IB.SOL}}{mg_{RPC1}\text{IB.RES}} \qquad \text{equ. 1}$$

where mg_{RPC1} *IB.SOL* is the mass of protein in the solubilzation solution of the IB's calculated from concentration measurement by RPC 1 method and volume of IB SOL solution. mg_{RPC1} *IB.RES* is the mass in the 20% IB suspension calculated from concentration measurement by RPC 1 method and volume of the solution after resuspension of the isolated IB's in DI.

Mass balance for refolding was calculated using equ.2 and is expressed as % recovery. IB's solubilized with either 4% NLS, 8 M urea+/- 5mM DTT or 6M GuHCl +/-5mM DTT were diluted and refolded as described in 3.3.1 and 3.3.2, respectively.

$$\% \ \text{Ref} = \frac{mg_{RPC1}\text{Ref.END}}{mg_{RPC1}\text{IB.SOL}} \qquad \text{equ. 2}$$

where mg $_{RPC1}$ Ref.END is the mass of protein according to concentration measurement with RP-HPLC found in the refolding solution times volume of refolding solution. mg $_{RPC1}$ IB.SOL is the mass of protein found in the IB solubilisate, % REF is the recovery after refolding.

Mass balance after solubilization with 4% NLS and subsequent refolding of all mutants as calculated by equations 1 and 2 are illustrated in Figure 3. Raw data can be found in Tables 6 and 7.

Table 6 shows the recovery after solubilization with NLS of the IB_RES suspension and recovery after refolding calculated from analytical method RPC I for all tested mutants. Also shown is the protein concentration in the IB-RES solution and protein concentration in the refolding solution determined by analytical method RPCI. Table 7 shows protein concentration determined by RPC I of IB_RES, IB_SOL and REF.END samples after solubilization with urea (SOL:urea) and solubilization with GuHCl (SOL:GuHCL).Refolding time was 96hrs at 4° C. The dilution factor was 500 and 50 for IB_RES and IB_SOL, respectively to yield the REF.END solution.

Table 6

Mutant #	c [mg/ml] IB_RES	Recovery solubilization (%)	c [mg/ml] ref.end	Recovery refolding (%)
MYC 138	27.2	110	1.18	72
MYC 123wt	54.9	138.75	1.65	99.2
MYC 140	18.7	105.55	1.46	82.8
MYC 137	19.2	150.25	1.83	83.7
MYC 139	19.9	138.66	1.82	84.2
MYC 119	13.6	152.27	1.59	84.84
MYC 118	19.8	136.1	1.77	89.8
MYC 136	15.1	141.5	1.76	90.1
MYC 133	16.3	117.175	2.09	94.1
MYC 135	11	135.65	1.82	96.3
MYC 134	35.9	77.35	2.73	99.1
MYC 106	13.7	124.75	2.95	87.1
MYC 130	19.4	87	1.84	92.9

Table 7

			SOL: urea	SOL: GuHCl
Mutant #	c IB_RES	c IB_SOL	c REF.end	c REF.end
MYC 118	19,8	2	0,091	0,075
MYC 119	13,6	3,46	0,07	0,056
MYC 130	19,4	2,94	0,044	0,026
MYC 133	16,3	1,34	0,023	0,0193
MYC 135	11	1,41	0,035	0,042

Mass balance of solubilization was exceeding 100% for 10 of the 12 investigated mutants. This could be due to the fact that the IB suspension was a crude sample type and eventually, the IB's were not completely dissolved when the sample was taken, leading to an inhomogeneous solution and thus underestimating total protein concentration. Data variability is high, with a relative standard deviation for 6 mutants analyzed twice ranging from 2.6% (Myc 138) to 42.9% (Myc 133).

% Recovery after refolding was between 72% (Myc 138) and 99% (Myc 134). The expected recovery is 100% (similar to recovery after solubilization). Recoveries after refolding were all lower than 100% and scatter not as much as for recovery after solubilization. This indicates that estimation of protein concentration in IB.SOL and REF.end samples is more accurate than in IB.RES samples. However, calculation of recovery primarily serves as a control for solubilization and refolding experiments.

Refolding yields related to the solubilization solution IB.SOL and the IB suspension IB.RES when 8M urea or 6M GuHCl was used as solubilization agent are shown in Figure 4.

The recovery varies from 44% to 230% reflecting the problems with measurement of protein concentration especially in IB.SOL and IB.RES samples, possibly due to insufficient homogenization prior to sampling.

Concentration in the REF.END samples after urea solubilization was comparable to concentration in REF.END after GuHCl solubilization (see Tables 6 and 7) though by an average factor of 1.2 higher. Dilution was thus consistent.

Example 6 - Solubilization time

Solubilization time with NLS was studied for all mutants. The time until the solution started to become clear and the time until no further clarification could be observed was recorded and is illustrated in Figure 5.

In contrast to using 2% NLS, solubilization with Urea +/- DTT and GuHCl +/-DTT was 2-3 times faster.

A correlation between the number of Cysteines and time required for clarification to start was found. With the exception of mutant 134 (5 cys), mutants with 4 cysteine residues solubilized faster than mutants with 5 cysteine residues. Figure 6 shows a variability chart where START [min] of clarification is plotted versus the 3 categories:

alignment of heavy or light chain fragment at the N-terminus, number of linker elements and cysteine residues. With exception of Myc 134, indicated with 1 in Figure 6, data points in category with 4 Cysteines scatter around earlier solubilization start times than compared with data points in the category with 5 cysteines.

A regression model (R^2= 0.72 when Myc 134 is excluded) predicted that start of solubilization would decrease from 20 min to 11.3 min for a Mycograb construct with 4 cysteines instead of 5 (model not shown).

Example 7 - RPC 2- NLS refolds

Mycograb REF.END samples solubilized and refolded according to Biomeva adapted process (described in Example 4) mutants were analyzed by RPC 2.

An overlay of REF.END samples from all investigated mutants including the wild type (MYC 123) is shown in Figure 7 and Figure 8. Mutant Myc 116 was screened earlier in lab DSP-DEV 1 and was included in the overlay for comparison reasons.

In Figure 7, chromatograms of REF.END samples generated from IB's isolated at bench scale and in Figure 8, chromatograms of REF.END samples generated from IB's isolated at larger scale in the pilot plant are shown.

Elution profiles were compared with respect to:

1. Retention time peak 1, reflecting hydrophobicity
2. Shape of peak 1, reflecting presence of dimer and homogeneity of monomer species when the peak is sharp
3. Ratio area of monomer/dimer peak (peak1) to aggregate/impurity peak (peak 2), reflecting aggregate/impurity content

Retention time peak 1, reflecting hydrophobicity

The retention time of the monomer/dimer peak for the tested mutants is listed in Table 8.

Table 8: Retention time [min] of peak 1 in RPC2 for the tested mutants and the molecule properties

Mutant	t_r min]	linker	cystein	chain orientation
MYC 130	ave: 8,16 n=3; RSD=4,6%	3X	4	VH
MYC 106 origami	9,8	3X	4	VH
MYC 138	9,99	5X	4	VH
MYC 139	10,035	6X	4	VH
MYC 123_Wt	10,041	3X	5	VH
MYC118	10,075	4X	5	VH
MYC 137	10,116	4X	4	VH
MYC 134	10,26	6X	5	VH
MYC 140	10,526	4X	4	VL
MYC 119	10,535	4X	5	VL
MYC 135	10,558	5X	5	VL
MYC 136	10,637	6X	4	VL
MYC 133	10,659	5X	5	VH

Retention time varies greatly with molecule construct. There is no trend of retention time (reflecting hydrophobicity) increasing with linker length, as would be expected. One linker element consists of four Glycines and one Serine residue. Glycine is hydrophobic in contrast to Serine, which is hydrophilic. However, the 4x higher Glycine content in the linker seems not to significantly increase the hydrophobicity as measured by retention time in RPC 2.

Retention time of Myc 130 is shorter than for the rest of the mutants. 10 amino acids were replaced by amino acids of more hydrophilic (5 serines) nature, thus decreasing the hydrophobicity of the molecule und consequently resulting in earlier retention time. Excluding this data point from statistical analysis results in a model showing significant difference in retention time when the orientation of the VL is N-terminal compared to an orientation when its C-terminal. Retention time increases by 0.41 min when the VL element is located N-terminal compared with when its located C terminal. Figure 9 shows the scaled estimates and a prediction profiler of the model with number of cysteines, linker length and chain fragment orientation as factors and retention time as response.

Note that retention time of Myc 130 was excluded from the model. A plot of retention time versus linker length, shown in Figure 10, demonstrates that retention time for this construct is lower compared to the rest of the mutants because of point mutations resulting in more hydrophilic nature, as mentioned above.

Shape of peak 1

It is assumed that a sharp peak 1 with no or only little shoulders reflects the homogeneity of a monomeric Mycograb. Peak shape was assessed by overlays of the RPC 2 chromatograms of REF.End samples from all mutants and sharpest peaks were determined for the mutants Myc 137, Myc 138 and Myc 139. Peak1 was sharper than the wild type Myc 123 and peaks 1 and 2 were almost base-line separated. This may be an indication that these constructs express the monomeric/dimeric protein with greater homogeneity than the wild type.

Ratio area of monomer/dimer peak

Impurity/aggregate content in relation to monomer/dimer was lowest for Myc 116 and MYC123 wt as shown in Figure 7 (Myc 123 wt). However, analysis of these two samples was performed 3 months earlier by another laboratory and an increase of peak 2 was noticed over time. A chromatogram of REF.End sample from Myc 123 that was prepared and analyzed in the same month as REF.End samples of the tested mutants is shown in Figure 8. Comparison of the chromatogram of MYC 123 shown in Figure 7 with that of shown in Figure 8 leads to the conclusion that sample preparation and analytical method are not exactly reproducible.

The area ratio of monomer/dimer peak to aggregate peak was determined by normalizing peak 1 to the same peak maximum. The peak area of peak 2 after normalization was ranked according to increasing size using visual area estimation. The normalized overview is shown in Figure 11.

The following ranking could be established:

Myc 116, Myc 139, Myc 136 < Myc 119, Myc 12, Myc140 < Myc137, Myc 135, Myc138 < Myc106 < Myc130 < Myc 134 < Myc118 < Myc133

RPC 2 chromatograms of REF.End samples generated from IBs of mutants Myc 106, 134, 136-139 were processed in the pilot plant and showed lower impurity /aggregate peaks (cf Figure 8) than IB's of mutants Myc 118-135 isolated at bench scale.

Example 8 RPC 2 urea/GuHCl refolds

Mutants Myc 118, 119, 130 and Myc 133 were dissolved with 7,6M Urea +/- DTT and 5,6M GuHCl +/-DTT and refolding was initiated by dilution in refolding buffer.

All REF.End samples did not show the monomer/dimer peak (peak 1) in RPC 2. A huge peak 2, assumed to be aggregates and impurities is predominant. A representative RP HPLC chromatogram of a refold end sample from mutant Myc 119 is given in Figure 12. The sample was prepared as described in Example 4.

The monomer was expected to elute at approximately 10.5 min. Peaks eluting earlier are not identified and were not observed in refolds with 0.5% NLS. The huge peak 2 indicates strong aggregation. Similar elution profiles were obtained for all REF.End samples after urea/GuHCl solubilization.

Similar elution profiles were obtained when the refold was done by 1:10 dilution with a buffer containing 20mM Tris/Base, 0.5M L-arginine and 2mM Cystin at pH 9.0. A representative chromatogram is shown in Figure 13.

The strong aggregation tendency was confirmed by SDS-Page under non reducing conditions, a huge and intense HMW smear was detected for a REF.End sample, with no monomeric band visible after urea solubilization. This smear then disappeared when the sample was reduced and a monomeric Mycograb band appeared. In Figure 14, SDS Page analysis of a reduced and non- reduced REF.End sample after urea solubilization is shown. Lanes 4-7 show REF.IM and REF.End sample of MYC 119 under non-reducing conditions at 2 different dilutions. Lanes 10-13 show the same sample under

reducing conditions. The aggregate smear disappeared when the sample was reduced and the monomeric as well as the dimeric band became visible.

Urea and GuHCl were present in the refolding solution at low concentration (0.14M in case of a 1:50 dilution and 0.72M in case of a 1:10 dilution for urea; for GuHCl, it was 0.11M in case of a 1:50 dilution and 0.56M in case of a 1:10 dilution) cannot prevent the protein from aggregation. Using DTT does not seem to have a significant effect on aggregation as RP-HPLC chromatograms (RPC 2) with and without DTT looked comparable, as shown in Figure 12.

In contrast to the REF.End sample, RPC 2 chromatogram of a IB.SOL sample dissolved with urea was comparable in terms of peak shape with a IB.SOL sample dissolved in 2% NLS. Figure 15 shows an overlay of HPLC chromatograms.

SDS-Page analysis of a non-reduced IB.SOL sample showed stronger HMW bands when the sample was dissolved with urea than in case of a IB.SOL sample dissolved in 2% NLS. This is shown in Figure 14: in lane 3, the sample was solubilized with urea and in lane 8, the sample was solubilized with 2% NLS. It can be concluded that peak 2 in RP-HPLC does not give an indication about aggregate content since peak 2 of an chromatogram overlay is even smaller for an IB.SOL sample in urea than in NLS.

Subsequent refolding did not yield a monomeric peak but the protein completely aggregates.

Example 9 - SDS-PAGE reducing and non-reducing

Reducing and non-reducing SDS-Page was performed to determine impurities and aggregates content in REF.End samples. With reducing SDS-Page Mycograb species appeared as monomeric and dimeric band and host cell impurity content in the sample could be distinguished from aggregated species when compared to a non-reducing SDS-Page gel. Non-reducing SDS Page showed Mycograb monomers, dimers and aggregates. Comparing a non-reduced SDS-Page silver stain gel with a reducing SDS-Page analysis, the amount of aggregated species could be evaluated semi-quantitatively.

Reducing and non-reducing SDS -Page gels of REF.END samples of all tested mutants are shown in Figure 16 and Figure 17.

The gel in Figure 16 on the left side shows a reducing SDS-Page of REF.End samples from mutants MYC 118, 119, 130, 133, 134, 135, 137. The band at 30kDa is monomeric Mycograb and it is predominant in all samples. According to the migration of the monomeric band, Mycograb expressed in mutant MYC 134 seems to have higher molecular weight than the other mutants analyzed on the gel. The same but to a lesser extend was detected for MYC 135. According to Table 9, MYC 134 has the highest theoretical molecular weight among the mutants shown in Figure 16, followed by MYC 135.

In the non reducing SDS-Page gel, aggregated species as well as the dimer are visible. In lanes 13 (MYC 134) and 15 (MYC 137), HMW bands are fainter than in the other lanes. This would indicate a lower content of aggregated species, however, also the bands for the monomer are more faint.

Double bands of different migration time and intensity in comparison with each other were observed for all mutants with exception of Myc 133 shown in Figure 16. Identification of these bands was hardly possible, however it was assumed that it was Mycograb Monomer of native like structure.

Figure 17 shows a reducing and non –reducing SDS Page of REF.End samples from mutants MYC 106, 136,138,139,140 and the wild type, MYC 123. Differences in MW for the different constructs could be determined according to different migration of the monomeric band. The bands in lanes 5 and 7 appeared at a slightly lower MW than the bands in lanes 2,3,6 and 4 which was in agreement with the theoretical MW listed in Table 9.

The mass of protein applied to the gel was not consistent; monomeric bands varied in intensity because protein concentration determination in the REF.End sample was not accurate enough. Thus semi-quanitative analysis of impurity content was not possible. However, the higher impurity content in REF.end sample of Myc 106 was obvious as

the thickness of the monomeric band was comparable to one of Myc 140 but the intensity of the other bands was much higher.

SDS-Page analysis indicated that a REF.End sample of Myc 106 $_{origami}$ contained more HCP and product related impurities. However, this was not confirmed by RPC2 analytical method, where the area of peak 2 was in the same range as for the other mutants.

Example 10 - SEC 0.5% NLS : Determination of molecular weight of Mycograb species in the REF. END sample

All REF.END samples prepared according to the adapted Biomeva process (see Example 4) were analyzed with SEC- HPLC in 0,5% NLS and the average molecular weight was determined. An overlay of the SEC chromatograms is shown in Figure 18 and Figure 19.

The average molecular weight ranged from 48.6kDa to 65.8 kDa. The broadness of the peaks reflects the heterogeneity of species in the sample. Though approximately 80% of the product was monomeric in REF.End samples, dimers and higher MW species were present as well as non-product related impurities, resulting in a broad elution peak.

In Figure 18, the elution profile of Myc 130 sticks out because of increased fronting compared with the other investigated samples. This might have been due to increased heterogeneity in the sample because of the construct's nature (more hydrophilic) or due to an accidentally different sample treatment.

Samples shown in Figure 18 were prepared simultaneously and stored at 4°C for 5 days prior to analysis. Samples shown in Figure 19 were prepared simultaneously and stored at 4°C over night days prior to analysis. Samples seemed to be stable at 4°C as MW of the constructs were in a similar range.

Fronting of Myc 130 may have beem due to higher amount of aggregated species compared with the other investigated samples. However, peak 2 in the corresponding

RPC 2 chromatogram was not outstandingly large but it has been observed that there is only sometimes a correlation between increased MW determined by SEC 0.5% NLS and large peak 2 peak area, determined with RPC2.

Additionally, SDS-page of Myc 130 did not indicate a higher impurity content and increased heterogeneity of the sample. Other factors leading to fronting in SEC such as column overloading and increased temperature during analysis can be excluded as all samples were analyzed on the same day.

There was a very early retention time for Myc 130 in RPC2.

It was assumed that the average MW in a REF.End sample was increasing for an increasing amount of dimers, aggregates and impurities. The calculated MW of a monomeric Mycograb expressed in the different mutants was between 26 and 27kDa because of the different linker length and other mutations. A table listing theoretical MW- calculated from the amino acids-, MW of the REF.End sample determined by SEC, # of amino acids, linker length and number of cysteines is given in Table 9.

Table 9: SEC-HPLC 0,5% NLS results of REF.End samples from all tested mutants. The mutants are ranked according to their theoretical molecular weight.

MUTANT	**MW theory**	**SEC**	**# aa**	**Linker length**	**Number of cysteines**
MYC 130	26.1	65.8	246	3X	4
MYC 118	26.4	48.6	251	4X	5
MYC 137	26.466	47.1	251	4X	4
MYC 119	26.5	49.9	252	4X	5
MYC 140	26.55	49.3	252	4X	4
MYC 133	26.7	57.9	256	5X	5
MYC 138	26.78	51.0	256	5X	4
MYC 135	26.8	52.2	257	5X	5
MYC 136	27.018	64.6	261	6X	5
MYC 134	27.062	62.6	261	6X	5
MYC 139	27.09	64.7	261	6X	4
MYC 123Wt	27.32	63.5	256	3X	5
MYC 106 origami	27.38	61.5	256	3X	4

Theoretical MW was plotted versus MW determined by SEC and a linear relationship with a correlation coefficient of 0.77 was found when the data point for Myc 130 was excluded.

Mutants Myc 106, Myc 138 and Myc 135, represented by dots to the right of the lower dashed line in Figure 20, had a lower average MW than the wild type. Mutants Myc 133, 136 and 139, represented by dots to the left of the higher dashed line had a higher average MW than the wild type. However, assay variability has to be taken into account. Additionally, from Figure 19 it can be seen that the mass of injected protein was not always the same as peak area for some of the samples. Formation of covalent aggregates should be decreased for mutants with 4 cysteines as compared with Mycograb with 5 cysteines because no free cysteine in mutants with 4 cysteines is available after formation of intermolecular SS bridges. Intermolecular covalent aggregates are formed during refolding even with a mutant with only 4 cysteines but it would be likely that the amount is lower than for a mutant with 5 cysteines.

Example 11 - SEC Formulation: NLS removal by UF/DF from refolding solution to measure aggregation tendency

In order to evaluate aggregation tendency of Mycograb mutants, the NLS concentration was lowered by an average factor of 5 from the REF.End solution with ultra/diafiltration using a stir cell. The total buffer volume used during diafiltration divided by the retentate volume is the diafactor and was 2.5.

The rationale of this experiment was to investigate aggregation tendency of the mutants when the dissolving agent NLS is lowered to a concentration at which aggregation cannot be prevented anymore.

It was assumed that formation of aggregates can be assessed with SEC-HPLC formulation. The elution buffer contained 0.5M urea buffer but no NLS to suppress aggregation.

Increase in molecular weight was used as a measure of tendency to aggregate and allows the mutants to be compared.

Mutants Myc 137, Myc 106, Myc 119 and the wild type Myc 123 were selected for a first set of experiments. Table10 shows the molecular weight (MW) in kDa determined by SEC- HPLC running in formulation buffer and concentration of NLS (%) determined by RP-HPLC for REF.End samples from mutants MYC 119,137,106 and MYC 123 before and after UFDF. The NLS reduction factor was calculated from NLS concentration in the sample prior to UFDF (# 2) divided by the concentration of NLS after UFDF (#3). The % increase MW based on SEC HPLC 0,5% NLS was calculated from the MW of the REF.END sample (#1) and the MW after UFDF (#3) for the respective Mutant.

Table 10

		MYC 123		MYC 119		MYC 106 origami		MYC 137	
Sample no.	Sample code	MW [kDa]	c NLS%	MW [kDa]	c NLS %	MW [kDa]	c NLS%	MW [kDa]	c NLS %
#1	REF. END*	63.5[**]	n.d	49.3 [**]	n.d	61.5 [kDa] **	n.d	47.1 [**]	n.d
#2	Prior to UFDF	214	0.52	359	0.58	202	0.607	212	0.57
#3	After YFDF	178	0.08	245	0.171	184	0.132	194	0.114
	NLS reduction factor		6.5		3.4		4.6		5
	% increase MW based on *	180%		397%		199%		312%	

*The molecular weight was determined with an analytical SEC HPLC method containing 0.% NLS in the running buffer (SEC HPLC 0.5% NLS).

** data from Table 9.

The apparent high molecular weight determined by SEC HPLC (running with formulation buffer) of the sample prior to UFDF was due to aggregation of protein during analysis.

The sample prior to UFDF still contained 0.5% NLS. As the sample migrated through the column, NLS was more strongly retarded than the protein and consequently aggregation occurred. Therefore this analytical method was not suited to determine molecular weight of samples containing NLS.

In order to determine molecular weight of Mycograb in the REF.End sample, SEC HPLC with 0.5% NLS in the running buffer was used. The increase in MW after removal of NLS in the REF.End sample by UFDF was calculated as described above in relation to Table 10. Values are shown in row 6 of Table 10. MYC 123 showed the smallest increase in MW whereas MYC 119 had the strongest increase, 400%.

MYC 123 has a lower aggregation tendency than MYC 119 based on these data. However, it has to be considered that the analytical SEC HPLC may have an influence on the protein structure and on the formation of aggregates. Moreover, increase of MW is calculated from data obtained from two different analytical methods and it cannot be assessed if the MW of a sample is similar when it is determined with the two different methods.

In Table 11, the mutants are ranked according to increase in MW after NLS removal together with the mutations. It has to be noted that the two mutants with a 3x linker element have significantly lower % of MW increase compared with mutants with a 4x linker element.

Table 11: Increase of molecular weight after removal of NLS by UFDF for the 4 investigated mutants.

MUTANT	%molecular weight increase	HIS tag	Linker length	VH-VL alignment	cysteins
MYC 123_Wt	180	YES	3X	VH N-terminal	5
MYC 106 origami	199	YES	3X	VH N-terminal	4
MYC 137	312	NO	4X	VH N-terminal	4
MYC 119	397	NO	4X	VL N-terminal	5

Figure 21 shows an overlay of the SEC HPLC chromatograms obtained from the sample prior to UFDF and after each volume reconstitution. The shape of the elution peaks did not significantly change with reduction of NLS concentration. Consequently, the MW of the sample also remained constant with reduction of NLS. This might be an indication that there is a limit in the concentration of surfactant below which aggregation is initiated but does not proceed further. However, the impact of the analytical method on the MW of the sample is not known and may be the reason why all samples have similar MW.

The NLS concentration was reduced on average (n=5) to a concentration of 0.124% after 5 volume reconstitutions, corresponding to a diavolume of 2.5. Theoretically, for a retention R = 0 of NLS, the calculated remaining NLS concentration should be 0.020%. Equation 3 was used for calculation of the theoretical remaining NLS concentration:

$$c_{retentat} = c_{feed} \times \left(e^{(R-1)\times[\ln VCF + N]}\right) \qquad \text{equ. 3}$$

where $c_{retentat}$ is the concentration of NLS in the retentate, c_{feed} is the concentration of NLS in the feed solution. R is the retention, the fraction of solute that is retained by the membrane. VCF is the volume concentration factor and N is the diavolume which is the total buffer volume introduced to the operation during diafiltration divided by the retentate volume.

The discrepancy between the theoretical and the measured concentration of NLS in the retentate is an indication for retention of NLS by the membrane greater than 0. This might be due to interaction between NLS and the protein, membrane and/or other components. The ability to deform can also cause unwanted retention.

Example 12 - Pep-Map analysis

REF.End samples of all mutants were analyzed for disulfide bridging with peptide map. Prior to analysis, the sample was alkylated with iodoacetamide, digested with trypsin and subjected to LC-MS. UV peaks of the single peptides were identified with mass spectrometry. The peptides with free SH groups, correctly and incorrectly formed S-S

bonds as well as dimeric peptides were, whenever possible, semi-quantitatively determined.

A correctly folded construct with 5 cysteines forms an S-S bond between Cys 23 and Cys 97 which corresponds to T3 and T9 respectively. The bond T3 –T9 is located in the light chain. The other disulfide bond is on the heavy chain between Cys 159 and Cys 224, corresponding to T12 and T17. The 5th cysteine is located at Cys28 and corresponds to the T4 peptide.

A construct with 4 cysteines always lacks the Cys 28 residue, the correct S-S bridges are similar as for a construct with 5 cysteines.

Mutants 118, 119, 130, 135, 133, 134, 137 and C28Y +HIS (106) as well as C28Y-HIS (108) were analyzed in lab AL1. Mutants 106 origami, 136, 138, 139, 140 and the wild type 123 were analyzed in analytical lab AL2 with a different device. The sensitivity of the mass spectrometer in AL1 is higher than that in AL2 and therefore, a semi-quantitative analysis of mutants analyzed in AL2 could not be obtained. However, it was possible to determine if correctly formed SS bridges are present.

A summary of the obtained data is given in Table 12. The results are compiled in 3 categories: free SH, bridged cysteines and dimeric cysteines. Free cysteines indicate a SH group that did not form a disulfide bond. Bridged cysteines are intramolecular disulfide bonds and dimeric cysteines represent intermolecular disulfides. W indicates that a weak signal for the respective peptide was detected and X represents peptides giving strong signals. Mutants analyzed in AL 2 are labeled with *.

Table 12: Pep Map analysis of all tested mutants

			Mutants with 5 cysteins						Mutants with 4 cysteins							
			Myc 118	Myc119	Myc 133	Myc 134	Myc 135	Myc 123*	Myc 106* origami	Myc 130	Myc 137	Myc 138*	Myc 139*	Myc 140*	Myc 106 (C28Y + HIS)	Myc 108 (C28Y - HIS)
Free cysteins	Free SH at T4		w	w	w				no T4!							
	All other free SH		w	w	w	w	w	X								
bridged cysteins	correct disulfides															
		T3-T9	w	w	w		w			X	X				X	X
		T12-T17	X	X	X						X				X	X
	incorrect disulfides															
		all other combinations	X	X	X	X	X	X	X	X	X	X	w	X	X	X
Dimers	T9-T9		X		w	w				X	w				w	w
	other dimers							w								

A 'correctly 'folded Mycograb with 5 cysteines should give a significant signal for free SH at the T4 peptide and no signal corresponding to other free SH groups. Additionally, a strong signal for the correct disulfides T3-T9 and T12-T17 is expected and incorrect SS bonds should not be present. Lastly, no intermolecular SS bonds should be present.

A 'correctly 'folded Mycograb with 4 cysteines should not have any free SH groups. Only the correct S-S bonds T3-T9 and T12-T17 should be detected. Additionally, no intermolecular SS bonds should be present.

Table 12 shows that neither the wild type nor any of the mutants gave strong signals for the correct S-S bonds only. It has to be considered that the REF.End sample consists of a population of differently folded and covalently aggregated species, so that a mixture of all possible combinations of disulfide bonds and free SH groups is present. However, a promising mutant should at least show significant signals for both of the correct S-S bridges which is the case for MYC 137 and the mutants C28Y+HIS and C28Y – HIS.

In 5 cases, only incorrect S-S bonds were found, where no, or only a weak, signal was obtained for the correct disulfide bonds.

Signals for MYC 123, MYC 138, MYC 139 and MYC 140 were extremely weak and reanalysis of the samples did not yield higher signals. Though Mycograb specific peptides were found, the cysteine containing peptides gave no or only a weak signal.

This might be due to ineffective digestion of the respective portion of the protein with trypsin because of structurally blocked cleaving sites. It was also noted that mutants with increased linker length (5 and 6X instead of 3X) were more difficult to digest and consequently signals for the late eluting peptides could not or could only hardly be detected.

Interestingly, covalent disulfides were, with one exception, only formed between the two T9 peptides, corresponding to Cys 97 residues.

Mutants Myc 118, 119, 130, 133, 137 and the mutants C28Y+HIS and C28Y – HIS gave stronger signals for correct S-S bonds than the wild type. However, it has to be considered that Myc 123 was analyzed with a different mass spectrometer of lower sensitivity and hence signal intensities cannot be compared. Signals from peptides of Myc 123 can be compared with signals from mutants 106 origami, 136, 138, 139 and 140. For none of these constructs, correct disulfide bridges were obtained. Only signals for incorrect disulfides were found.

Pep Map results for Myc 137, Myc C28Y+HIS and Myc C28Y – HIS were most promising with significant signals for both correct disulfide bridges. Mutants Myc 118, Myc 119 and Myc 133 also showed a certain amount of native like disulfide bridging, however, the signal for T12-T17 was weak.

Mutant Myc 130 showed strong signals for the T12-T17 SS bond, but the second correct disulfide was not found.

Conclusions

Correlations of analytical results

The best recoveries after refolding were obtained with the MYC 123 (wt) and MYC 134, as determined by the titer assay with Poros column (RPC1).

The solubilization of IBs with chaotropic agents was faster than solubilization with NLS. RPC 2 chromatograms of IBs solubilized with urea or NLS were comparable. However, SDS Page indicated a stronger dissolving power of NLS as the aggregate smear was reduced compared with IBs solubilized with urea, see Figure 14.

Refolding after solubilization with chaotropic agents by dilution and use of different additives such as Cysteine, L-Arginine, 1% NLS and low concentration of urea/ GuHCl did not show a monomeric peak in RPC 2, see Figure 12 and Figure 13. The protein completely aggregated which was confirmed by SDS Page, non reducing and the RPC 2 chromatogram showed a huge peak 2.

The determination of MW with SEC HPLC 0.5% NLS correlated with an R^2 of 0.77 with the theoretically calculated MW when 1 outlier was excluded. The resolving power of SDS PAGE was not sufficient to detect all subtle differences in MW, but a migration time difference was seen between two mutants of a MW differing by 0.6kDa in theory and 14kDa as measured with SEC HPLC (MYC 134 and MYC 118, respectively).

RPC 2 chromatography was able to confirm decreased hydrophobicity of a mutant where 10 hydrophobic amino acids were replaced by more hydrophilic ones (MYC 130). The linker element did not have easy access to binding sites of the stationary phase and did not therefore have influence on retention behavior. It was also observed that Pep Map analysis for constructs with increased linker length gave weaker signals for the peptides of interest compared with constructs with shorter linker. It appeared that the linker element was not easily accessible for the digesting enzyme.

RPC2 chromatograms for MYC 137,138 and 139 showed the sharpest peak which is attributed to a Mycograb Monomer compared with the other tested mutants, indicating increased homogeneity of the sample.

Pep Map analysis showed that almost no free SH group was present in REF.End samples for mutants with 4 Cysteines. This indicates an almost complete formation of disulfide bridges (incorrect and correct ones) as well as formation of covalent aggregates. For mutants with 5 cysteines, weak signals were obtained for various free

SH groups but only mutants Myc 118, Myc 119 and Myc 133 had a free SH group at T4, the location of the 5th cysteine which should remain reduced in the 'native' monomer. This is an indication that the SH group on the T4 peptide preferably forms SS bonds because all mutants with 5 cysteines gave signals for free SH groups but only in 3 of them a free SH group at T4 was detected.

MW determined with SEC HPLC is very dependent on the buffer matrix in the sample. A couple of SEC HPLC methods had to be established with a running buffer similar to the buffer of the sample. The matrix dependency of MW determination makes comparison of MW across process steps difficult (Table 10).

The UFDF experiment showed that NLS is to some extent retained in the sample solution and cannot efficiently be removed.

The results obtained with Pep Map of Mutants MYC C28Y +HIS, C28Y-HIS and MYC 137 were particularly promising. The results indicate that a mutant with a HIS tag and only 4 cysteines is particularly preferred. The HIS tag is required for purification with IMAC, a purification step of high efficiency. A construct with only 4 cysteines is more likely to form correct disulfides and fewer covalent aggregates.

Effects of mutations

The most beneficial effect of the mutations can be attributed to the removal of the 5th cysteine. The number of correct disulfide bonds was increased compared with constructs with 5 cysteines. Additionally, solubility of the IBs was enhanced compared with constructs with 5 cysteines.

Exchanging the orientation of the heavy and light chain fragment had a minor effect on retention time in RPC 2 where the retention time decreased when the VL element was C terminal compared with an N-terminal orientation.

The tendency of the peptides to aggregate after NLS removal may be increased with the number of linker elements.

CLAIMS:

1. An scFv peptide comprising a V_H domain and a V_L domain linked by an amino acid spacer, wherein the V_H domain comprises a sequence with at least 80% sequence identity to the sequence of SEQ ID NO. 64 and the V_L domain comprises a sequence with at least 80% sequence identity to the sequence of SEQ ID NO. 66 and wherein the scFv peptide comprises the substitution or deletion of an amino acid in the V_H domain at the position corresponding to C_{28}.

2. An scFv peptide according to claim 1 wherein the substitution of the amino acid in the V_H domain is $C_{28}Y$.

3. An scFv peptide according to claim 1 or 2 wherein the amino acid spacer comprises the sequence $(GGGGS)_n$.

4. An scFv peptide according to any one of the preceding claims further comprising a purification tag, preferably a sequence of 6 histidine residues at the C-terminus.

5. An scFv peptide according to any one of the preceding claims comprising an amino acid sequence selected from the group consisting of SEQ ID NO. 8, 10, 14, 18 and 22 wherein Xaa denotes an amino acid residue other than cysteine and wherein the N-terminal methionine residue may optionally be cleaved off, preferably wherein Xaa denotes a tyrosine residue.

6. A nucleic acid molecule comprising a sequence encoding an scFv peptide according to any one of the preceding claims.

7. A nucleic acid molecule according to claim 6 further comprising the sequence $(taa)_n$ located at the 3' end of the sequence encoding the scFv peptide wherein n is 1 or 2.

8. A nucleic acid molecule according to claim 6 or 7 wherein the molecule comprises a sequence selected from the group consisting of: SEQ ID NO. 7, 9, 13, 17 and 21 wherein nnn denotes a codon coding for an amino acid other than a cysteine residue.

9. A nucleic acid molecule according to any one of claims 6 to 8 wherein the molecule is a DNA or RNA molecule.

10. A pharmaceutical composition comprising an scFv peptide according to any one of claims 1 to 5 in combination with a pharmaceutically acceptable excipient, diluent or carrier.

11. A combined preparation comprising an scFv peptide according to any one of claims 1 to 5 and an antifungal agent or an anticancer agent.

12. A composition comprising an scFv peptide according to any one of claims 1 to 5 and an antifungal agent or an anticancer agent.

13. A method of treating a patient with a fungal infection comprising administering to the patient an effective amount of an scFv peptide according to any one of claims 1 to 5.

14. A method according to claim 13 further comprising the step of administering to the patient an effective amount of an antifungal agent.

15. An scFv peptide according to any one of claims 1 to 5 for use in the treatment of a fungal infection.

16. The method according to claim 14 or 15 or the scFv peptide according to claim 15 wherein the fungal infection is due to a Candida, Cryptococcus, Histoplasma, Aspergillus, Torulopsis, Mucormycosis, Blastomycosis, Coccidioidomycosis or Paracoccidioidomycosis organism.

17. The combined preparation according to claim 11, the composition according to claim 12, the method according to claim 13, 14 or 16 or the scFv peptide according to claim 15 or 16 wherein the antifungal agent is an azole antifungal, preferably selected from the group consisting of: itraconazole, voriconazole isavuconazole, fluconazole, miconazole, ketoconazole, and posaconazole.

18. The combined preparation according to claim 11, the composition according to claim 12, the method according to claim 13, 14 or 16 or the scFv peptide according to claim 14 or 16 wherein the antifungal agent is selected from the group consisting of: a polyene antifungal agent and an echinocandin antifungal agent.

19. The combined preparation, composition, method or scFv peptide according to claim 18 wherein the polyene antifungal agent comprises amphotericin B or a derivative thereof.

20. A method of treating a patient with a cancer disease comprising administering to the patient in need an effective amount of a scFv peptide according to any one of claims 1 to 5.

21. A method according to claim 20 further comprising the step of administering to the patient an effective amount of at least one other anticancer agent.

22. An scFv peptide according to any one of claims 1 to 5 for use in the treatment of a cancer disease.

23. The combined preparation according to claim 11, the composition according to claim 12, the method according to claim 20 or 21 or the scFv peptide according to claim 22 wherein the anticancer agent is selected from the group consisting of: Doxorubicin, Daunorubicin, Epirubicin, Herceptin, Docetaxel, Cisplatin, Imatinib, Paclitaxel, Docetaxel, Hydroxyurea, 5-fluorouracil, Oxaliplatin, Irinotecan, Cytarabine and Raltitrexed.

24. A method of treating a patient with of a condition involving raised levels of TNFα and/or IL-6 comprising administering to the patient an effective amount of a scFv peptide according to any one of claims 1 to 5.

25. An scFv peptide according to any one of claims 1 to 5 for use in the treatment of a condition involving raised levels of TNFα and/or IL-6.

26. A method according to claim 24 or an scFv peptide according to claim 25 wherein the condition involving raised levels of TNFα and/or IL-6 is selected from the group consisting of: sepsis, SIRS and an autoimmune disease.

27. A method or scFv peptide according to claim 26 wherein the autoimmune disease is selected from the group consisting of: Crohn's disease, rheumatoid arthritis, ulcerative colitis and systemic lupus erythematosus.

28. A vector molecule comprising a nucleotide sequence according to any one of claims 6 to 9.

29. A host cell comprising a vector molecule according to claim 28.

30. A method for producing a scFv peptide according to any one of claims 1 to 5 which comprises culturing a host cell having incorporated therein an expression vector containing, under control of suitable transcriptional control elements, a nucleic acid molecule according to any one of claims 6 to 9 under conditions sufficient for expression of said peptides in the host cell thereby causing the production of said peptide and recovering the peptide produced by said cell.