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(56) Documents Cited:
EP 1283057 A **WO 2009/030780 A**
WO 1997/004312 A
Journal of Molecular Biology (1998); Vol 281, pp 379-391, "From synthetic coiled coils...", Ghirlanda et al

(58) Field of Search:
INT CL **C07K**
Other: **WPI, EPODOC, TXTE, BIOSIS, MEDLINE**

(54) Title of the Invention: **Parathyroid hormone related protein antagonists**
Abstract Title: **Parathyroid hormone related protein antagonists**

(57) A single-chain triple-stranded alpha-helical coiled coil polypeptide binds to parathyroid hormone related protein (PTHrP) and can act as an antagonist of it. This PTHrP-binding "alpha-body" may be used for the treatment of cancer, hypercalcemia associated with cancer and cancer metastasis. Nucleic acids encoding said alpha-bodies, host cells comprising said nucleic acids, as well as pharmaceutical compositions comprising said alpha-bodies are provided. The alpha-bodies are preferably selected by phage display of a coiled-coil scaffold.

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FIGURE 1/6

scLib013_L16_AC11 (groove binding)

ANCHOR	a d a d a d a d		
N	| | | | | | | |	MKYLLPTAAAGLLLLAAQPA (PelB)	*20*
a1	MSIEEIQKxIAxIQExIAxIQKxIYxMT		*28*
L1		GGSGGGSGGGSGGGSG	*16*
a2	MSIEEIQKQIAAIQKQIAAIQKQIYAMT		*28*
L2		GGSGGGSGGGSGGGSG	*16*
a3	MSIEEIQKQIxAIxEQIxAIxKQIxAMTP		*29*
C		GGSGGAAAHHHHHHGRAE@	*18*
PIII		(PIII) - (ph)	

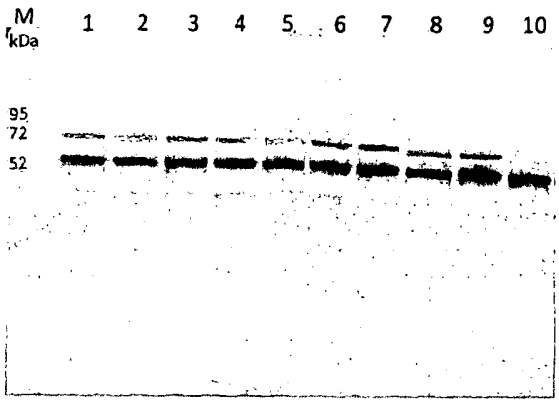
scLib140_L14_AC7 (groove binding)

ANCHOR	a d a d a d		
N	| | | | | |	MKYLLPTAAAGLLLLAAQPA (PelB)	*20*
a1	MDIQQIQKxIAxIQExIYxMT		*28*
L1		GGSGGGSGGGSGGG	*14*
a2	MDIQQIQKQIAAIQKQIYAMT		*28*
L2		GGSGGGSGGGSGGG	*14*
a3	MDIQQIQKQIxAIxEQIxAMTP@		*29*
C		GGSGGAAAHHHHHHGRAE@	*18*
PIII		(PIII) - (ph)	

scLib013_L16_C9 (surface binding)

ANCHOR	a d a d a d a d		
N	| | | | | | | |	MKYLLPTAAAGLLLLAAQPA (PelB)	*20*
a1	MSIEEIQKQIAAIQKQIAAIQKQIYAMT		*28*
L1		GGSGGGSGGGSGGGSG	*16*
a2	MSIEEIQKQIAAIQKQIAAIQKQIYAMT		*28*
L2		GGSGGGSGGGSGGGSG	*16*
a3	MSIEEIQxQIxxIQxQIxxIQxQIxxMTP@		*29*
C		GGSGGAAAHHHHHHGRAE@	*18*
PIII		(PIII) - (ph)	

FIGURE 2/6



Lanes 1 to 6: scLib013_L16_AC11

Lanes 6 & 7: scLib013_L16_C9

Lanes 8 & 9: scLib140_L14_AC7

Lanes 10: wt phage (no fusion protein)

FIGURE 3/6

scLib013_L16_LBC8 :

ANCHOR	a d a d a d a d		
N	| | | | | | | |	MKYLLPTAAAGLLLLAAQPA (PelB)	*20*
a1	MSIEEIQKQIAAIQKQIAAIQKQIYRMT		*28*
L1		GGSGGGSGGGSGGGSG	*16*
a2	MSIEEIQKQIAAIQKQIAAIQKQIYRMT		*28*
L2		$GGSGX_8GGSG$	*16*
a3	MSIEEIQKQIAAIQKQIAAIQKQIYRMTP@		*29*
C		GGSGGAAAHHHHHHGRAE@	*18*
PIII		(PIII)-(ph)	

scLib140_L14_LBC14 :

ANCHOR	a d a d a d		
N	| | | | | |	MKYLLPTAAAGLLLLAAQPA (PelB)	*20*
a1	MDIQQIQKQIAAIQKQIYRMT		*28*
L1		GGSGGGSGGGSGGG	*14*
a2	MDIQQIQKQIAAIQKQIYRMT		*28*
L2		X_{14}	*14*
a3	MDIQQIQKQIAAIQKQIYRMTP@		*29*
C		GGSGGAAAHHHHHHGRAE@	*18*
PIII		(PIII)-(ph)	

scLibISSI_L16_LBCAlt :

ANCHOR	a d a d a d a d		
HT1	| | | | | | | |	MGHHHHHHHHHHSSGHIEGRH	21
a1	MSIEEIQKQISSIQKQISSIQKQIYRMT		28
L1		GGSGGGSGGGSGGGSG	16
a2	MSIEEIQKQISSIQKQISSIQKQIYRMT		28
L2		$(G/S, X)_7$ G/S G	16
a3	MSIEEIQKQISSIQKQISSIQKQIYRMTP@		29

FIGURE 4/6

Lib. name	Number of res mutated (type of randomization)	Mutation location	Lib. size
scLib013_L16_C9	**9** (NNK, customized)	helix C (res. b, c, f)	$1.0x10^8$
scLib013_L16_AC11	**11** (NNK, customized)	helix A (res g, c) & helix C (res b,e)	$1.4 x10^8$
scLib140_L14_AC7	**7** (NNK, customized)	helix A (res g, c) & helix C (res b,e)	$1.7x10^8$
scLib013_L16_LBC8	**8** (NNK)	linker LBC (middle res)	$1.2x10^9$
scLib140_L14_LBC14	**14** (NNK)	linker LBC (all res)	$1.2x10^9$
scLibISSI_L16_LBCAlt	**15** $([RGT,NNK]_7,RGT)$	linker LBC (alternated res)	$1.0x10^9$

FIGURE 5/6

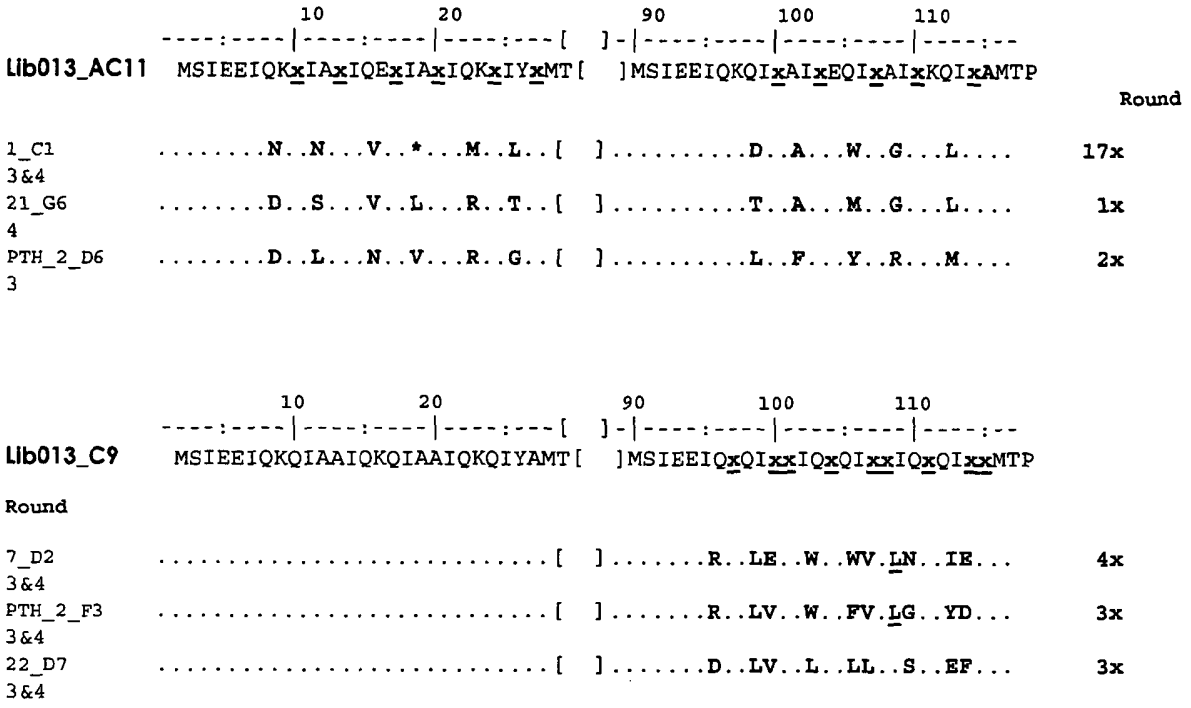
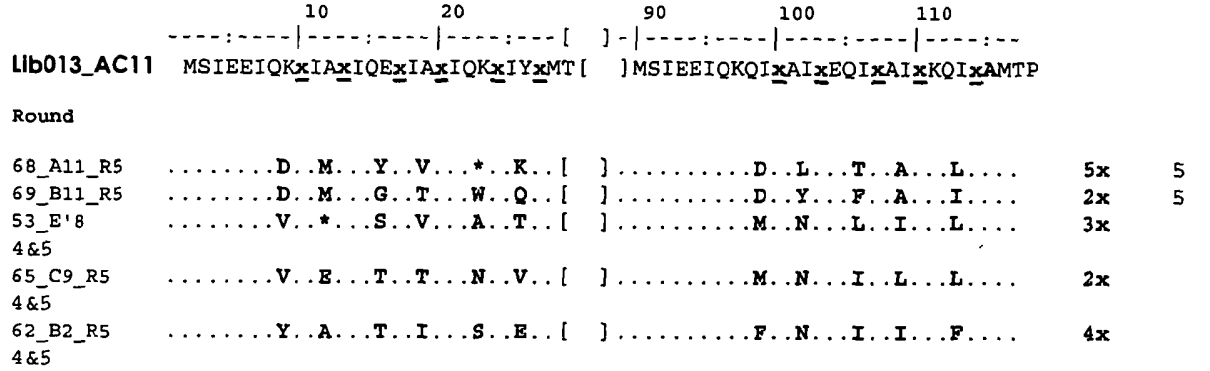

10 20 90 100 110
Lib013_AC11 MSIEEIQKxIAxIQExIAxIQKxIYxMT[ ]MSIEEIQKQIxAIxEQIxAIxKQIxAMTP
Round
1_C1 .....N..N...V..*...M..L..[ ].....D..A...W..G...L.... 17x
3&4
21_G6 .....D..S...V..L...R..T..[ ].....T..A...M..G...L.... 1x
4
PTH_2_D6 .....D..L...N..V...R..G..[ ].....L..F...Y..R...M.... 2x
3
10 20 90 100 110
Lib013_C9 MSIEEIQKQIAAIQKQIAAIQKQIYAMT[ ]MSIEEIQxQIxxIQxQIxxIQxQIxxMTP
Round
7_D2 .....[ ].....R..LE..W..WV.LN..IE... 4x
3&4
PTH_2_F3 .....[ ].....R..LV..W..FV.LG..YD... 3x
3&4
22_D7 .....[ ].....D..LV..L..LL..S..EF... 3x
3&4

FIGURE 6/6


10
20
90
100
110
Lib013_AC11 MSIEEIQKxIAxIQExIAxIQKxIYxMT[ ]MSIEEIQKQIxAIxEQIxAIxKQIxAMTP
Round
68_A11_R5 .....D..M...Y..V...*..K..[ ].....D..L...T..A...L.... 5x 5
69_B11_R5 .....D..M...G..T...W..Q..[ ].....D..Y...F..A...I.... 2x 5
53_E'8 .....V..*...S..V...A..T..[ ].....M..N...L..I...L.... 3x
4&5
65_C9_R5 .....V..E...T..T...N..V..[ ].....M..N...I..L...L.... 2x
4&5
62_B2_R5 .....Y..A...T..I...S..E..[ ].....F..N...I..I...F.... 4x
4&5

PARATHYROID HORMONE RELATED PROTEIN ANTAGONISTS

Technical Field

Parathyroid hormone-related protein (PTHrP) is a protein member of the parathyroid hormone family. Apart from its normal functions in the regulation of endochondral bone development and mammary gland formation, it is also involved in the development of cancer. Increased PTHrP expression by a number of tumors is responsible for hypercalcemia of malignancy. In addition to its role in metastasis (e.g. bone metastasis in lung cancer), PTHrP has been shown to exert anti-apoptotic activities in cancer cells. The present invention relates to human Parathyroid Hormone-Related Protein (PTHrP), isoforms thereof and antagonists thereto in the diagnosis and treatment of a disease or a disease related condition, particularly cancer and hypercalcemia.

BACKGROUND OF THE INVENTION

PTHrP is related in function to the "normal" parathyroid hormone (PTH). Whereas the main developmental roles for PTH are associated with chondrogenesis, PTHrP has additional effects on cell survival and proliferation, and on epithelial-mesenchymal interactions. When a tumor secretes PTHrP, this can lead to hypercalcemia. As this is sometimes the first sign of malignancy, hypercalcemia caused by PTHrP is considered a paraneoplastic phenomenon. The majority of patients with advanced cancer and hypercalcemia have been shown to have elevated circulating levels of PTHrP with or without associated osteolytic skeletal metastasis. PTHrP has been shown to regulate tumor-relevant genes and is suggested to play a role in tumorigenesis, modulation of tumor progression and response to treatment. A very recent study shows that PTHrP affects both the extrinsic and intrinsic apoptosis signaling pathways and thus confers chemoresistance (Gagiannis *et al.*, Int J Cancer *in press*).

Tumors are known to develop from normal cells through a series of transformations. Activation of signaling molecules and in particular growth factor related pathways could lead to malignant transformation of normal cells. Cancer mortality can be linked to the ability of tumors to undergo metastatic spread. The spread of tumors from the original site and the ability to home in specific tissues likely involves multiple steps as tumors are progressing from a non-invasive to an invasive state.

PTHrP is associated with the great majority of malignancies in the context of hypercalcemia, including breast, colon, skin, renal and lung cancer as well as hematological malignancies such as lymphomas, leukemias and multiple myelomas. Importantly, elevated PTHrP expression has been shown in these tumors in the absence of hypercalcemia and of elevated levels of circulating PTHrP. PTHrP correlates with the metastatic process in several types of cancer including breast, prostate and colon cancer and may be a prognostic indicator in cancer patients. Several studies suggest that PTHrP stimulates bone metastasis in vivo (Deng et al., Clin Exp Metastasis 2007, 24:107-119; Iguchi et al., Cancer Res 1996, Liao et al., Int J Cancer 2008). The mechanism underlying PTHrP stimulation of bone metastasis is believed to be indirect by activating osteoclastic bone resorption and the release of local growth factors within the bone microenvironment that in turn stimulate growth of tumor cells within bone. Currently, the main treatment of bone metastasis focuses on the reduction of osteoclastic activity by the class of agents known as bisphosphonates. PTHrP inhibition has therefore been identified as a potential target to inhibit osteoclastic activity within bone by reducing PTHrP production of tumor cells within bone. Humanized monoclonal antibodies directed at the N-terminal end of PTHrP have been generated and shown to be effective in nude mice models of hypercalcemia and bone metastasis. In patients with osteolytic bone metastasis, humanized monoclonal anti-PTHrP antibodies directed to the N-

terminus have entered clinical trials (Chugai Pharmaceutical Co.), but these were discontinued in phase II.

In addition to its indirect effect on the bone metastatic process, several studies suggest that PTHrP may directly affect the growth and invasive abilities of tumor cells. Most of these studies were conducted in vitro and tend to indicate that PTHrP stimulates invasion and migration in different cell lines including breast, prostate and melanoma. In vivo data aside from studies on bone metastasis are very limited. One study indicates that PTHrP may be responsible for the growth of renal cancers and that growth and potentially also metastasis is reduced by the administration of an antibody directed towards the N-terminal end of PTHrP in nude mice transplanted with a human renal cancer cell line (Talon et al., Carcinogenesis 2006).

Alternative mRNA splicing of the human PTHrP gene gives rise to three isoforms of 139, 141, 173 amino acids, bearing the same N-terminal part (in the present manuscript, these isoforms are referred to as PTHrP 1-139, PTHrP 1-141 and PTHrP 1-173). There is strong sequence homology between species but alternate splicing has not been reported in the lower species except for the canine gene. The mouse, rat, rabbit, bovine and chicken genes may only give rise to the PTHrP 1-139. There is considerable divergence among species in the C-terminal end of PTHrP beyond amino acid 111. The long isoform, PTHrP 1-173 may be unique to humans but its function is currently unknown although it has been suggested to play a role in cartilage growth.

PTHrP undergoes a proteolytic processing, possibly tissue-specific, into smaller bioactive forms, comprising an N-terminal peptide, a midregion peptide and C-terminal peptides. The physiologic midregion secretory form is PTHrP 38-94, which is endowed with pro-apoptotic activities.

Because the PTH-like activity of PTHrP resides within the N-terminal portion of the molecule, studies have used N-terminal

fragments for in vitro and in vivo studies, particularly for studies of the PTH/PTHrP receptor Type-1 (PTHR1) which can be activated by both PTH 1-34 and PTHrP 1-34. PTHR1 belongs to the G-protein coupled class of receptors (GPCRs) that feature 7 trans-membrane domains linked to G-proteins. Ligand binding to PTHR1 results in the activation of both adenylate cyclase (cAMP pathway) and phospholipase C (PLC). PTHR1 is ubiquitously expressed in most tissues, but increased expression in cancer cells has been observed.

Apart from the N-terminal region of PTHrP involved in PTHR1 binding, other PTHrP regions must be involved in binding events as well. Evidence exists that heat shock protein-70 (HSP70) expressed on the surface of cancer cells binds PTHrP fragments 1-34, 37-86, 109-141 and 140-173 with cytoprotective consequences *in vivo* (Grzesiak *et al.*, Endocrinology 2005). Both PTHrP 1-34 as PTHrP 107-139 inhibit the activation of caspases induced by chemotherapeutic agents (Gagiannis *et al.*, Int J Cancer 2009). PTHrP 140-173 has been shown to exert anti-apoptotic effects and to bind various intracellular proteins involved in cytoprotection (Grzesiak *et al.*, Peptides 2006).
PTHrP region 88-106 is a nuclear localization sequence that targets various PTHrP fragments to the cell nucleus. For example, it has been shown that PTHrP 67-101, which is translocated to the nucleus, stimulates growth in breast cancer cells, whereas PTHrP 67-86, which remains cytosolic, does not (Kumari *et al.*, Int J Cancer 2006).

PTHrP and PTHR1 also appear to have a role in the myocardium of patients affected by different cardiac degenerative processes (Monego et al., Basic Res Cardiol 2009).

Taken together, it is clear that PTHrP is a versatile protein that exerts a variety of functions via molecular interactions that involve different regions of the PTHrP molecule. In this respect, it may be beneficiary to target various parts of the PTHrP molecule simultaneously, in order to tackle the various roles of PTHrP in disease.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Amino acid sequence of the cc-domain libraries. Mutations relative to parent scABs are also underlined.The x positions are encoded by NNK codons that were synthesized with a nucleotide mixture of N at 1st position = 40%A, 12%C, 16%G, 32%T, N at 2nd position = 28%A, 24%C, 20%G,28%T, K at 3rd position = 50%G, 50%T in case of scLib013_L16_AC11 and N at 1^{st} and 2^{nd} positions = 10%C, 30%A, 30%G, 30%T and K at 3rd position = 50%G, 50%T for scLib140_L14_AC7.The NNK codons for scLib013_L16_C9 were N at 1^{st} and 2^{nd} positions = 10%C, 30%A, 30%G, 30%T and K at 3rd position = 50%G, 50%T.

Figure 2: Western-Blot analysis of the different cc-domain library phages ($2x10^{11}$ phages/lane; several batches of phages from each library were tested). Samples were blotted onto nitrocellulose membrane after SDS-PAGE in reducing conditions. The presence of the fusion product was demonstrated by using a mouse anti-gpIII antibody followed by an AP-anti-mouse conjugate and the blot was developed by adding the NBT-BCIP substrate.

Figure 3: Amino acid sequence of the Loop libraries. The X positions are encoded by NNK codons that were synthesized with a nucleotide mixture of N at 1^{st} and 2^{nd} positions = 25%C, 25%A, 25%G, 25%T and K at 3rd position = 50%G, 50%T; G/S states for a 1:1 mixture of Gly and Ser.

Figure 4: Overview of the currently available scAB libraries.

Figure 5: Sequence alignment of scAB-phage clones specific for the PTH peptide. Only the relevant part of the alpha-body sequences is given, with x corresponding to randomized positions. For each sequence, the occurrence and selection

round(s) are specified. *: glutamic acid residue (suppressed TAG stop codon), L: non-intended point mutation.

Figure 6: Sequence alignment of scAB-phage clones specific for the PTHrP peptide. Same representation as in Figure 5.

SUMMARY OF THE INVENTION

An embodiment of the present invention is the use of PTHrP or its isoforms as a diagnostic agent and treatment for disease, including several types of cancer, and disease related conditions, such as hypercalcemia of malignancy and cachexia.

A further embodiment of the present invention is inhibition of PTHrP or its isoforms, to treat tumor growth and metastatic spread thereof in several types of cancer.

A further embodiment of the present invention is inhibition of PTHrP or its isoforms in the myocardium.

Another embodiment of the present invention is directed to a particular class of proteinaceous molecules - termed alpha-bodies - directed against PTHrP. One aspect of the present invention is alpha-bodies, directed against the N-terminal part of PTHrP (PTHrP 1-34), that inhibit its binding to or signalling via the PTHR1 receptor.

Another aspect of the present invention is alpha-bodies directed at a part of PTHrP that is not the N-terminal part, said part being more preferably amino acid residues 38 to 94, but equally preferably amino acid residues 88 to 106, but equally preferably amino acid residues 107 to 139, and equally preferably amino acid residues 140 to 173.

A further aspect of the present invention is alpha-bodies directed against the N-terminal part of PHTrP (PTHrP 1-34) and

which simultaneously target another part of PTHrP, said part being more preferably the amino acid residues 38 to 94, but equally preferably amino acid residues 88 to 106, but equally preferably amino acid residues 107 to 139, and equally preferably amino acid residues 140 to 173, with the objectives to I) potentially obtain higher affinities, II) inhibit both PTHR1-mediated signalling events as well as other signalling events.

A further aspect of the present invention is alpha-bodies directed simultaneously against two parts of PHTrP other than the N-terminus, said parts being more preferably the amino acid residues 38 to 94, but equally preferably amino acid residues 88 to 106, but equally preferably amino acid residues 107 to 139, and equally preferably amino acid residues 140 to 173, with the objectives to I) potentially obtain higher affinities, II) inhibit signalling events beyond PTHR1-mediated signalling events, while retaining PTHR1-mediated events.

Another embodiment of the present invention is directed to the development of specific assays for the detection of specific PTHrP isoforms or in vivo generated proteolytic fragments thereof, including, but not limited to, sandwich assays such as IRMA, ELISA and chemiluminescent assays. Preferably, the alpha-bodies described herein are used to detect these isoforms.

Another embodiment of the present invention is determining which tumors express which PTHrP isoform(s) or in vivo generated proteolytic fragments thereof, in order to enhance disease treatment. Preferably, the alpha-bodies described herein are used.

Another embodiment of the present invention relates to the effect of PTHrP on cell growth and metastasis, and more specifically the reduction of tumor growth and/or metastasis following the targeting by alpha-bodies of one of various PTHrP fragments or of one of various combinations thereof.

A further embodiment of the present invention is a method of inhibiting the growth, metastasis and invasion of tumor cells by administering to a patient a therapeutically effective amount (e.g. an amount that eliminates or reduces the patient's tumor burden) of alpha-bodies of the present invention. The alpha-bodies of the present invention can be administered via different routes (e.g. parenteral, oral, sublingual, topical). Parenteral administration can occur either subcutaneously, intramuscularly, intravenously or within the tumor itself.

Another embodiment of the present invention is directed to derivatives of the alpha-bodies of the present invention including, but not limited to (a) labeled (e.g. radiolabeled, enzyme-labeled or fluorochrome labeled) alpha-bodies of the present invention, for diagnosing or detecting tumors and tumor spread (e.g. metastasis) using known imaging technologies; and (b) toxin conjugates of the alpha-bodies of the present invention, where the alpha-bodies are conjugated to cytotoxic, radioactive or radiolabeled moieties for therapeutic ablation.

Further embodiments of the present invention are directed to an isolated alpha-body that specifically binds the N-terminal part of PTHrP (PTHrP 1-34), wherein said alpha-body can be linked to a diagnostic or therapeutic agent.

Further embodiments of the present invention are directed to an isolated alpha-body that specifically binds the midregion of PTHrP (PTHrP 38-94), wherein said alpha-body can be linked to a diagnostic or therapeutic agent.

Further embodiments of the present invention are directed to an isolated alpha-body that specifically binds the nuclear localization sequence of PTHrP (PTHrP 88-106), wherein said alpha-body can be linked to a diagnostic or therapeutic agent.

Further embodiments of the present invention are directed to an isolated alpha-body that specifically binds the C-terminal part of the PTHrP 1-139 isoform (PTHrP 107-139), wherein said alpha-body can be linked to a diagnostic or therapeutic agent.

Further embodiments of the present invention are directed to an isolated alpha-body that specifically binds the C-terminal part of the PTHrP 1-173 isoform (PTHrP 140-173), wherein said alpha-body can be linked to a diagnostic or therapeutic agent.

Further embodiments of the present invention are directed to an isolated alpha-body that specifically and simultaneously binds the N-terminal part of PTHrP and another PTHrP part that is not the N-terminal part, wherein said alpha-body can be linked to a diagnostic or therapeutic agent.

Further embodiments of the present invention are directed to an isolated alpha-body that specifically and simultaneously binds two PTHrP parts, neither of which is the N-terminal part, wherein said alpha-body can be linked to a diagnostic or therapeutic agent.

Further embodiments of the present invention are directed to the use of the D-isomer of PTHrP or a fragment thereof (contrary to the naturally occurring L-isoform) for the screening or biopanning of a natural L-isomeric alpha-body library. A D-isomeric PTHrP binding L-isomeric alpha-body emerging from such a screening or biopanning can then be used to construct a D-isomeric alpha-body with the same sequence, directed against natural L-isomeric PTHrP. D-isomeric alpha-bodies benefit in vivo from their resistance to proteolytic cleavage and potentially from altered pharmacokinetics.

A further embodiment of the present invention is directed to methods for producing an alpha-body, comprising: a) the design of a library (e.g. a phage library), b) the generation of a large set of alpha-body variants; c) either the screening or the biopanning of said library against a desired PTHrP isoform or isolated fragment derived thereof; d) following biopanning,

isolating and selecting at least one clone emerging from said biopanning.

A further embodiment of the present invention is directed to methods for treating hypercalcemia of malignancy, the method comprising administering to a subject in need of such treatment an effective amount of an isolated alpha-body that specifically binds PTHrP.

A further embodiment of the present invention is directed to methods for treating growth, metastasis or invasion of cancer cells, the method comprising administering to a subject in need of such treatment an effective amount of an isolated alpha-body that specifically binds PTHrP.

A further aspect of the present invention is directed to a method for diagnosing disease activity or metastatic spread of cancer cells, preferably prior to the development of hypercalcemia.

A further aspect of the present invention is directed to a method for treating disease, the method comprising administering to a subject in need of such treatment an effective amount of an alpha-body that specifically binds with PTHrP. In a preferred embodiment, the cancer cells can be selected from the group consisting of breast, lung, prostate, kidney, melanoma and squamous cancer cells.

A further embodiment of the present invention relates to a pharmaceutical composition comprising a PTHrP-binding alpha body as defined above.

Another embodiment of the present invention relates to a diagnostic agent comprising a PTHrP-binding alpha body as defined above.

Another embodiment of the present invention relates to the use of the alpha-body as defined for the manufacture of a medicament or a diagnostic reagent for the treatment or

diagnosis of any of the above indications associated with expression or secretion of PTHrP, preferably in the treatment or diagnosis of cancer, hypercalcemia or cachexia associated with cancer, inhibition of metastasis or invasion of cancer cells.

In a still further embodiment, the invention relates to a vector suitable for expression of the alpha-bodies of the invention in a host cell.

Another embodiment of the invention is a host cell transfected or transformed with a vector comprising the nucleic acid sequence encoding the alpha bodies of the invention and which is capable of expressing the alpha-bodies.

Another embodiment of the invention relates to a nucleic acid molecule comprising a nucleic acid sequence encoding the amino acid sequence of the alpha-bodies of the present invention.

The present invention also relates to methods of producing the alpha-body proteins, for example in transformed or transfected cells or cell lines (e.g. bacterial cells, mammalian cells or cell lines such as CHO cells, insect cells etc.), expressing the alpha-bodies of the invention, harvesting and purifying the alpha-bodies according to methods known in the art.

DETAILED DESCRIPTION OF THE INVENTION

In this disclosure, a number of terms and abbreviations are used. The following definitions of such terms and abbreviations are provided.

As used herein, a person skilled in the relevant art can generally understand that the term "parathyroid hormone-related protein" or its abbreviation "PTHrP" refers to the protein PTHrP or one of its isoforms, individually or collectively. In reference to one of the various isoforms according to the present invention, the isoform can be

referred to by the abbreviation PTHrP followed by the number of amino acid residues provided in that isoform. For example, the isoform comprising 139 amino acid residues can be referred to as PTHrP 1-139.

As used herein, a person skilled in the relevant art may generally understand the term "comprising" to generally mean the presence of the stated features or components as referred to in the claims, but that it does not preclude the presence or addition of one or more other features, components or groups thereof.

As used herein, a person skilled in the relevant art may generally understand the term "treatment" to generally refer to an approach for obtaining beneficial or desired results. Beneficial or desired results can include, but are not limited to, prevention or prophylaxis, alleviation or amelioration of one or more symptoms or conditions, diminishment of the extent of a disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

As used herein, a person skilled in the relevant art may generally understand the term "therapeutically effective amount" to be an amount sufficient to effect treatment when administered to a subject in need of treatment. In the case of the embodiments of the present invention, a therapeutically effective amount can include, but is not limited to, an amount that eliminates or reduces the effects of the disease, such as for example the tumor burden, in a subject.

As used herein, a person skilled in the relevant art may generally understand the term "amino acid sequence" to refer to an amino acid sequence of a naturally or non-naturally occurring protein molecule, "amino acid sequence" and like

terms, such as "polypeptide" or "protein", are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule. Amino acid sequences can be referred to as having an amino (N) terminus and a carboxyl (C) terminus. Individual amino acids in a peptide or polypeptide can be referred to as "residues" and such residues are numbered sequentially beginning from the N-terminus and increasing towards the C-terminus. The amino acids located generally proximal to the N-terminus are generally referred to as the N-terminal amino acids while those located generally proximal to the C-terminus are referred to as the C-terminal amino acids. It will be understood by a person skilled in the relevant art that the reference to amino acid residues as either N terminal or C-terminal amino acid residues may vary depending on the protein. It will be understood by a person skilled in the relevant art, that generally the N-terminal part of PTHrP extends generally from amino acid residues 1 to 34, the middle part or midregion extends generally from amino acid residue 38 to 94, the nuclear localization sequence extends generally from amino acid residue 88 to 106 and the C-terminal part generally starts at amino acid residue 107 until the end of the amino acid chain.

A person skilled in the relevant art may generally understand that a gene may produce multiple RNA species that are generated by differential or alternative splicing of the primary RNA transcript. The polypeptides transcribed from these multiple RNA species are referred to as "isoforms". Isoforms can be expressed in different tissues of the same organism as a result of, for example, alternative splicing of RNA. Alternatively, isoforms can be encoded by different genes.

The term "fragment" as used herein in reference to single chain amino acid sequences refers to a polypeptide that may have an amino (N) terminus portion and/or carboxy (C) terminus portion deleted as compared to the native protein, but wherein

the remaining amino acid sequence of the fragment is identical to the amino acid sequence of the native protein.

The term "naturally-occurring" or "native" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been modified is naturally-occurring.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule that is expressed from a recombinant DNA molecule.

Numerous techniques that are well known in the art are used to detect binding to PTHrP or fragments thereof in association with the present invention. These techniques include, but are not limited to RIA (radioimmunoassays), ELISA (enzyme-linked immuno-sorbent assays), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitation reactions, immunodiffusion assays, Western blots, precipitation reactions, agglutination assays (e.g. gel agglutination assays, hemagglutination assays, etc.), complement fixation assays, immunofluorescence assays, protein A assays, immunoelectrophoresis assays, etc.

As used herein, the term "Western blot" refers to the analysis of protein(s) (or polypeptides) immobilized onto a support such as nitrocellulose or a membrane. The proteins are run on acrylamide gels to separate the proteins, followed by transfer of the protein from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized proteins are then exposed to antibodies with reactivity against an antigen of interest. The binding of the alpha-bodies may be detected by various methods, including the use of radiolabeled antibodies, enzyme linked antibodies, etc.

The term "transgene" as used herein refers to a foreign, heterologous, or autologous gene that is introduced into a

cell, cell line or organism. The term "foreign gene" refers to any nucleic acid sequence that is introduced by experimental manipulations and may include an autologous gene. The term "autologous gene" may encompass variants (e.g. polymorphisms or mutants) of the naturally occurring gene.

As used herein, the term "vector" is used in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another.

The alpha-bodies of the present invention can be synthesized using chemical synthesis methods known in the art. Alternatively, the alpha-bodies of the present invention can be produced by genetic engineering techniques. Thus, the invention relates to a nucleic acid, for example, DNA or RNA, encoding an alpha-body of the present invention; an expression vector comprising said nucleic acid; a host cell transformed or infected with said nucleic acid or expression vector as well as a method for the production of an alpha-body of the invention comprising transforming or infecting a host cell with the nucleic acid according to the invention, preferably the vector according to the invention.

It may thus be understood by a person skilled in the relevant art that alpha-bodies of the present invention can be made by recombinant DNA methods. DNA encoding the alpha-bodies of the invention can be readily synthesized using conventional procedures. Once prepared, the DNA can be placed into expression vectors, which are then transformed or transfected into host cells such as E. coli or P. pastoris, in order to obtain the synthesis of alpha-bodies in the recombinant host cells.

Thus, the invention also relates to a method for the production of an alpha-body of the invention comprising transforming, transfecting or infecting a host cell with the

nucleic acid according to the invention, preferably the vector according to the invention, more preferably an expression vector according to the invention.

The invention also relates to a vector, preferably an expression vector, comprising said nucleic acid encoding an alpha-body of the present invention.

The term "expression vector" as used herein refers to a recombinant nucleic acid molecule that contains a desired nucleic acid target sequence and appropriate nucleic acid sequences necessary for the expression of nucleic acid or amino acid sequence in a host. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

The invention further relates to a host cell transformed or infected with said nucleic acid, vector or expression vector.

As used herein, the term "host" or "host cell" refers to any eukaryotic or prokaryotic cell (e.g. bacterial cells such as E. coli, yeast cells, mammalian cells, avian cells, amphibian cells, plant cells, fish cells, and insect cells), whether located in vitro or in vivo. For example, host cells may be located in a transgenic animal.

The terms "transformation" and "transfection" as used herein refer to the introduction of foreign DNA into respectively prokaryotic and eukaryotic cells. These procedures may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics.

As used herein, the terms "antagonist" and "antagonistic" refer to or describe a molecule which is capable of, directly or indirectly, substantially counteracting, reducing or inhibiting at least one of the biological activities of PTHrP, its isoforms or isolated proteolytic bioactive fragments thereof. Such antagonistic activity may result from the binding of PTHrP fragments 1-34, 38-94, 88-106, 107-139, or 140-173, or of any combination thereof. The PTHrP antagonist can also be a compound inhibiting PTHrP signaling or signaling of one if its isoforms or of one of its proteolytic bioactive fragments. The term PTHrP antagonist can be understood in its broad sense and includes any compound that decreases the biological effects of PTHrP or one of its isoforms or proteolytic bioactive fragments thereof.

As used herein, the term "alpha-body" is used in the broadest sense and specifically covers single anti-PTHrP alpha-bodies (including antagonist, and neutralizing or blocking alpha-bodies) and anti-PTHrP alpha-body compositions with specificity towards more than one epitope on PTHrP. Various procedures known within the art may be used for the selection of alpha-bodies directed against a specific antigen, or against derivatives, fragments, analogs, homologs or orthologs thereof. Such procedures can entail the use standard screening techniques, but also of recombinant display technology (e.g. phage display, bacterial display, yeast display, ribosome display, mRNA display).

All embodiments of the present invention relate to single-chain coiled coil molecules which are herein collectively denoted "alpha-bodies". Similar single-chain coiled coils have been described in Desmet et al., EP 08172017.9 and Desmet et al., US 61/120,642. Briefly, an alpha-body shall herein mean a single-chain coiled coil having a single contiguous amino acid chain with the formula HRS1-L1-HRS2-L2-HRS3, optionally supplemented with N- and C-terminal extensions resulting in the formula N-HRS1-L1-HRS2-L2-HRS3-C, wherein (a) each of HRS1, HRS2 and HRS3 is independently a heptad repeat sequence

(HRS), consisting of 2 to 7 consecutive heptad repeat (HR) units, which sequence can be designated as a-b-c-d-e-f-g, at least 50% of all heptad a- and d-positions are occupied by isoleucine residues, and HRS1, HRS2 and HRS3 together constitute a 3-stranded alpha-helical coiled-coil structure; (b) each of L1 and L2 are independently a linker fragment, covalently connecting HRS1 to HRS2 and HRS2 to HRS3, respectively, starting and ending with a proline or glycine, and consisting of 3 to 30 amino acid residues of which at least 50% are selected from the group proline, glycine, serine; and (c) N and C are independently an optional extension, covalently connected to the N- and C-terminal end of HRS1 and HRS3, respectively, this connection being marked by a helix-breaking proline or glycine.

As stated above, at least 50% of all heptad a- and d-positions are occupied by isoleucine residues. The remaining a- and d-positions can be any of the 20 naturally occurring amino acids, or non-naturally occurring amino acids.

Furthermore, the amino acids in each of L1 and/or L2 that are not proline, glycine, or serine can be any of the 20 naturally occurring amino acids, or non-naturally occurring amino acids.

Amino acids at positions b, c, e, f and g can also be any of the 20 naturally occurring amino acids, or non-naturally occurring amino acids.

The term "naturally occurring amino acid" refers to the following amino acids: alanine, aspartic acid, asparagine, cysteine, glutamine, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, proline, arginine, serine, threonine, valine, tryptophan, and tyrosine.

The term "non-naturally occurring amino acid" as used herein, refers to amino acids having a side chain that does not occur in the naturally occurring L-amino acids. Examples of non-natural amino acids and derivatives include, but are not limited to, agmatine, (S)-2-amino-4-((2-amino)pyrimidinyl)butanoic acid, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 4-amino-3-hydroxy-6-methylheptanoic acid, 6-aminohexanoic acid, alpha-aminoisobutyric acid, benzophenone, t-butylglycine, citrulline, cyclohexylalanine, desamino tyrosine, L-(4-guanidino)phenylalanine, homoarginine, homocysteine, homoserine, homolysine, n-formyl tryptophan, norleucine, norvaline, phenylglycine, (S)-4-piperidyl-N-amidino)glycine, ornithine, parabenzoyl-L-phenylalanine, sarcosine, statine, 2-thienyl alanine, and/or D-isomers of the naturally or non-naturally occurring amino acids.

Alpha-bodies are relatively small in size (about 10 to 20 kDa). Accordingly, this property is in agreement with the need for therapeutic protein molecules of a size that is smaller than an antibody or 5-helix. Alpha-bodies are also highly thermostable and are relatively insensitive to changes in pH and to proteolytic degradation. These properties form a solid basis for the development of engineered alpha-bodies with preservation of desirable physico-chemical properties and with acquired therapeutic functions. Therefore, alpha-bodies are in agreement with the need for therapeutic molecules that have a long shelf life. Alpha-bodies are also highly soluble, which is in agreement with the need for therapeutic molecules that can be easily tested in vitro. Most importantly, the fact that alpha-bodies are highly engineerable (substitutable, mutatable) is in agreement with the need for generating novel therapeutic molecules with high-affinity and specificity for selected target molecules.

In general, alpha-bodies are well suited as scaffold molecules for target recognition, for they are relatively insensitive to multiple simultaneous amino acid substitutions. For example, the structural integrity of an alpha-body is in general not substantially affected when all amino acid residues of a single groove are simultaneously mutated. Similarly, the structural integrity does not substantially change when all surface-exposed amino acid residues of a single alpha-helix are simultaneously mutated.

In a preferred embodiment, alpha-bodies are provided wherein the binding to PTHrP is characterized by a dissociation constant (Kd) or half maximal effective concentration (EC50) in the submicromolar range, preferably a dissociation constant (Kd) or half maximal effective concentration (EC50) of less than 1.0 micromolar, or the subnanomolar range, preferably a dissociation constant (Kd) or half maximal effective concentration (EC50) of less than 1.0 nanomolar, or the subpicomolar range, preferably a dissociation constant (Kd) or half maximal effective concentration (EC50) of less than 1.0 picomolar. These techniques include, but are not limited to RIA (radioimmunoassays), ELISA (enzyme-linked immunosorbent assays), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitation reactions, immunodiffusion assays, Western blots, precipitation reactions, agglutination assays (e.g. gel agglutination assays, hemagglutination assays, etc.), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc.

Hence, the present invention relates to an alpha-body which binds to PTHrP, preferably to at least one of the fragments of PTHrP mentioned above. The amino acid sequences of various isoforms are depicted in the sequence listing in SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3.

Since alpha-bodies are of non-human origin (i.e. they are synthetic), they are potentially immunogenic in humans and thus can give rise to an undesirable immune response. De-immunization involves the modification of potential helper T-cell epitopes that bind to MHC class II molecules in order to decrease any undesirable immune response (Van Walle *et al.*, Expert Opin Biol Ther 2007). Contrary to immunogenicity, antigenicity relates to the structural recognition by antibodies of an antigenic site on an antigen that is referred to as a structural epitope or a B-cell epitope. The robustness of the alpha-body scaffold allows the modification of a potential antigenic site on the surface of the protein without affecting its structural integrity to a great extend.

It will be understood by a person skilled in the relevant art that modifications of the alpha-bodies of the present invention are contemplated herein. The alpha-bodies of the present invention may be modified by conjugating, tagging or labeling through methods known in the art, to any known diagnostic or therapeutic agent, including but not limited to cytotoxic agents (e.g. immunotoxin conjugates), prodrugs, drugs (e.g. pharmaceutically active substances) or other effector molecules which are effective in the treatment of disease as well as known reporter molecules. Such modified alpha-bodies include, but are not limited to (a) labeled (e.g. radiolabeled, enzyme-labeled, fluorochrome or chemiluminescent compound) alpha-bodies of the present invention, for diagnosing or detecting tumors and tumor spread (e.g. metastasis) using known imaging technologies and (b) immunotoxin conjugates of the alpha-bodies of the present invention, where the alpha-bodies of the present invention are conjugated to known cytotoxic, radioactive, radiolabeled, prodrug or drug moieties (e.g. radioimmunotherapy). It will be understood by a person skilled in the relevant art that the term "cytotoxic agent", "cytotoxins" or "cytotoxic" as used herein generally refer to a substance that inhibits or prevents the function of cells and/or causes destruction of cells and includes, but is not limited to, radioactive

isotopes, chemotherapeutic agents, and toxins such as small molecule toxins or proteinaceous toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof. It will also be understood by a person skilled in the relevant art that the term "prodrug" as used in this application generally refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to target cells compared to the pharmaceutically active substance and is capable of being activated or converted into the more pharmaceutically active substance.

The binding specificity of alpha-bodies of the current invention is determined by an in vitro binding assay, such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the alpha-bodies can, for example, be determined by Scatchard analysis, Friquet analysis, surface plasmon resonance or isothermal titration. It is advantageous to identify alpha-bodies having a high degree of specificity and a high binding affinity for the target antigen.

It may be understood by a person skilled in the relevant art that alpha-bodies of the present invention can be made by recombinant DNA methods. DNA encoding the alpha-bodies of the invention can be readily synthesized using conventional procedures. Once prepared, the DNA can be placed into expression vectors, which are then transformed or transfected into host cells such as E. coli or P. pastoris, in order to obtain the synthesis of alpha-bodies in the recombinant host cells.

It will be understood by a person skilled in the relevant art that the compositions of the present invention, including but not limited to alpha-bodies, can be formulated into pharmaceutical compositions for administration in a manner customary for administration of such materials using standard pharmaceutical formulation chemistries and methodologies. It will also be understood by a person skilled in the relevant art that such pharmaceutical compositions may include one or

more excipients, carriers, stabilizers or other pharmaceutically inactive compounds, such as, but not, limited to, wetting or emulsifying agents, pH buffering substances and the like. Pharmaceutically acceptable salts can also be included therein.

The alpha-bodies of the present invention may be administered parenterally, including, but not limited to, intramuscular, intravenous, subcutaneous or intraperitoneal injection or infusion, and via transdermal or transmucosal administration. Alternatively, administration of the alpha-bodies of the present invention may be topical, including, but not limited to dermal, oral, anal or vaginal administration. The therapeutically effective doses may vary according to body weight and the timing and duration of administration will be determined by specific clinical research protocols.

The present invention is directed to the diagnosis, treatment and inhibition of tumor growth and its progression to metastatic sites through the inhibition of PTHrP signaling as a treatment for disease, including several types of cancers. More specifically, the present invention is directed to methods of inhibiting the anti-apoptotic activity of PTHrP which is exerted by both PTHR1-dependent and PTHR1-independent mechanisms (Gagiannis *et al.*, Int J Cancer 2009). Thus, a preferred alpha-body counteracts both anti-apoptotic mechanisms by binding to different PTHrP fragments simultaneously. Such fragments include but are not limited to amino acids 1-34, 38-94, 88-106, 107-139 and 140-173 of human PTHrP. The invention is also directed to in vivo imaging and therapeutic targeting of tumors and metastatic sites expressing and secreting PTHrP or proteolytic bioactive fragments thereof, using alpha-bodies thereagainst, such alpha-bodies being preferably tagged or labeled with a diagnostic (e.g. a reporter molecule) or therapeutic agent (e.g. cytotoxic agent, prodrug or drug). The invention is also directed to the detection of isoforms of PTHrP or of proteolytic bioactive fragments thereof as indicators of disease activity or metastatic spread, preferably prior to the

development of hypercalcemia, or as prognostic indicators of possible treatments. The invention may be applicable to many disease states, including but not limited to several types of cancer (such as breast, lung, prostate, melanoma and squamous of several types) expressing these isoforms, alone or in combination with other therapeutic agents.

EXAMPLES

EXAMPLE 1. Phage Display Libraries

Phage display is chosen as the method for high-throughput identification of - alpha-body binders, because it still is one of the most robust and versatile selection techniques available.
Initial experiments already demonstrate the feasibility to make fusions of an alpha-body with the gpIII coat protein of M13 phages in a suitable phagemid vector which results in a "3+3" monovalent display format (Smith GP, 1993. *Gene* 128, 1-2). The fusion construct starts with a PelB leader sequence attached to the alpha-body which is connected by a linker sequence, that contains an amber codon and a His_6-tag, to the gpIII protein. It is further shown that the alpha-body-gpIII fusion can be displayed by a fully infectious phage and that the soluble alpha-body can be expressed and purified in a non-suppressor strain.

The focus of the first libraries was to scrutinize the binding capabilities of the triple coiled coil (cc) itself, and further called cc-domain libraries. Thus, unbiased random libraries where unbiased is to be understood as "devoid of preferential binding features", in the groove between two of the three helices (A and C) were created as well as a "surface binder" with one randomized helix (C). In the mean time a second type of libraries has been designed which exploits the loop regions of the alpha-body (see below). All libraries were ordered at Geneart; they were delivered as transformed *E. coli* TG1 cells with a guaranteed minimum of 10^8 and 10^9 clones for the cc-domain and loop libraries, respectively.

EXAMPLE 2. Library Design

cc-domain libraries

The designs were based on the highly stable scAB013_L16 (SEQ ID NO: 4) and scAB140_L14 (SEQ ID NO: 5) reference alpha-bodies ('scAB' stands for single chain alpha-body). scAB140_L14 is the "short" version based on scAB013 wherein the third heptad of the coiled coil is removed. Variable residues were selected using the 3-D model for scAB013_L16. We tried to avoid excessive global charge and local hydrophobic patches. At the DNA level, the codon usage was chosen so as to reduce Pro and Gly to acceptable levels. To fully explore the scaffold binding potential, mutations were either introduced in one of the helices or in one of the grooves between two helices, thereby generating either "surface" or "groove" libraries. Full amino acid sequences of the cc-domain libraries are given in Figure 1.

Libraries designed for groove binding (scLib013_L16_AC11 and scLib140_L14_AC7)

Groove residues are located at c- and g-positions in the "left" helix A and at b- and e-positions in the "right" helix C. There are 14 such positions in the reference alpha-body scAB013 and 10 in scAB140. For both alpha-bodies, three of these were chosen *not* to be varied because of possible interference with N-terminal capping: a1c_E (a-chain, heptad 1, c-position, glutamate) stabilizes the backbone and helix dipole, position c1b_E stabilizes the helix dipole and points away from the other randomized residues while position c1g_Q covers the capping Met and also points away. The remaining 11 (resp. 7) positions were chosen to be varied in an identical way (same codon usage). In addition, two other modifications to standard scABs were made: (1) the "middle" Lys's (a2f_K and c2f_K) are mutated into Glu to break the crown of positive charges surrounding the randomized residues and (2) the C-

terminal Arg's at the 4c- (resp. 3c-) positions are mutated into Ala (as in scAB151) for the same reason *and* to eliminate a possible trypsin cleavage site.

Library designed for surface binding (scLib013_L16_C9): Helix C is the most exposed helix in an alpha-body because the preceding linker is kept away from it as a result of specific interactions near the turns. The most protruding residues are located at b-, c- and f-positions. Hence, we could in principle include 11 variable positions in the library. However, the b and c of the first heptad were excluded for the same reasons as in the cc-domain libraries. Thus, the C9 library comprises 9 variable positions. Similarly, the "middle" lysines and C-terminal arginines were mutated into glutamic acids and alanines, respectively.

The level of display on phages for these libraries was checked on Western-Blotting and found to be high (Figure 2). Densitometric analysis shows that in general 1/3 phages display an alpha-body.

Loop libraries

Here, we took advantage of the presence of flexible linkers in the scaffold to generate a conceptually different and complementary type of library. Indeed, the incorporation of mutations in the linkers should lead to randomized flexible loops which can give access to binding modes not reachable in the context of the groove and surface libraries described above. Alpha-bodies in the anti-parallel orientation have one loop on each side of the coiled coil, thus next to the N-terminus and C-terminus, respectively. Although the two linkers are in principal identical, we have decided to randomize the second one (*i.e.* the linker between helices B and C, referred to as LBC, located near the N-terminus) in order to prevent possible obstruction of binding due to the

proximity of the C-terminal fused gpIII of the M13 phage. In future libraries we will also randomize the first linker region.

Two libraries were designed with adjacent randomized residues. scLib013_L16_LBC8 wherein the 8 middle residues of the L16 BC-loop are randomized and scLib140_L14_LBC14 where the complete L14 is randomized. A third library contains a more subtle change where the 7 randomized residues each alternate with a Ser or Gly residue. Due to the specificities of the peptidic bond, alternating residues are more likely to face the same side and are thus spatially directed to contribute to binding. In the sequence, (G/S, X)7 G/S stands for a 7-time repeat of a pair where the first residue is either a Gly or a Ser (encoded by an RGT codon) followed by a randomized residue (encoded by NNK; the repeat itself is followed by another RGT codon).

Thus, six scAB phage-display libraries were designed and prepared (Overview in Figure 4). Given the unique structural features of the scaffold, two conceptually different types of randomization, namely in the coiled-coil domain itself and in the unstructured linkers between helices, could be designed. This enables different binding modes (via pockets, grooves between helices, helix surfaces or flexible loops) and will give access to a vast variety of structurally different targets.
The cc-domain scAB libraries were shown to be well-displayed on phage (Figure 2) and they have already been successfully used for the selection of peptide-binders (see Example 3).

EXAMPLE 3. scAB's targeting the N-terminal part of PTHrP.

The aim of the present example is to demonstrate a practically feasible method to generate alpha-bodies that specifically

bind PTHrP. Thus, the method was applied for the N-terminal part of PTHrP, as well as the homologous part of PTH.

Phage display technology was applied to search for binders to PTH and PTHrP. An equal mixture of phage prepared from the three scAB naïve libraries, scAB013_L16_AC11, scAB013_L16_C9 and scAB140_L14_C7 (see detailed description in Example 1) was used as input for selection against biotinylated peptides corresponding to the biologically active N-terminal 34-residue fragments of PTH and PTHrP :

PTH : Biotin-GGSG-SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF-NH_2

PTHrP : Biotin-GGSG-AVSEHQLLHDKGKSIQDLRRRFFLHHLIAEIHTA-NH_2

We adopted a standard soluble biopanning protocol. Briefly, phage were incubated with the target for 1.5 to 2 hours and then captured on streptavidin magnetic beads for 15 to 30 minutes. Mock experiments where the target was omitted were always performed in parallel. Bound phage were eluted with an acidic pH shock after washing of the beads. Four and five selection rounds were performed on the PTH and PTHrP peptides, respectively. The selection stringency - kept constant during of the different rounds - was as follows:

- amount of input phage: ~1.35 x 10^{12} viral particles
- 500 nM target; 50 μl streptavidin-coated magnetic beads
- 10 washes (10 x 1 ml 0.05% Tween-20-containing buffer)

Biopanning

For both target peptides, the ratio between the amounts of phage eluted from the target compared to the mock evolved from about 20 to 40 after the first round to more than 1000 in the final round. Whereas in case of PTH the enrichment factor reached about 350 after the 4^{th} round, it remained relatively modest (about 10) for PTHrP (data not shown).

The pools of phages recovered after the rounds 1 to 4 compared to the unselected input phage were analyzed by ELISA. The results confirmed the successful selection of phage populations specific for PTH and PTHrP emerging after the 3rd and 4th round, respectively. The later emergence of PTHrP-specific phages is in agreement with the observation of smaller enrichment factors and has motivated the completion of an additional 5th round on this target.

Screening of individual clones

Individual peptide-binder phage were identified by screening about 200 randomly picked colonies selected on the PTH peptide and 300 selected on the PTHrP peptides. In addition to phage from the last rounds, a small percentage of unselected and early rounds clones were also included in the sampling for signal comparison and internal controls. The phage were rescued in 96-well plates and directly used in ELISA's without any purification/quantification. All clones were tested for binding to both targets.

For phage selected on the PTH peptide, positive clones started to appear after the 3rd round, as could be anticipated from the results obtained on the pools. Moreover, most of the clones giving the highest signals displayed a good specificity for the PTH *versus* the PTHrP target.

For phage selected on PTHrP, some clones displaying signals above the background were also found after the 3rd round, but their positivity and frequency were much smaller than observed for the phage selected on the PTH peptide, which is in line with the modest enrichment factor observed during the biopanning. Moreover, most of the (slightly) positive clones from round 3 were cross-reacting with the homologous PTH target. The situation improved in the next rounds with the recovery of strongly positive PTHrP-specific phage.

The results obtained in the screening format on crude phage as shown above were confirmed on PEG-precipitated phage. For each target, a sample of phage from 10 positive colonies was

rescued, PEG-precipitated (2x), quantified and tested on ELISA for specific binding to the selection target. Biotinylated targets were immobilized at 100 nM on neutravidin coated Maxisorp plate; skimmed milk was used as blocking agent and an anti-M13-HRP conjugate was used for ELISA development. All phage (7.5×10^{10} phage particles per well) were tested on their selection target and checked for potential cross-reactivity towards the homologous hormone as well as towards an unrelated peptide and milk. The two unrelated scAB-displaying phage clones (negctrl 1 and negctrl 2) were negative on both targets and controls, ruling out potential artifacts due to the phage format. As expected, whereas high specific signals were obtained for the majority of the PTH-selected phage, specific binders were mostly found amongst the population retrieved from the 4^{th} round in case of the PTHrP-selected phage, which in the latter case reinforced the motivation to perform an additional round.

Sequence analysis

Sequence analysis of the selected scAB phage showed that virtually all the positive *and* specific clones were from the "long alpha-body" type with three full heptads (these correspond to the scLib013_L16_AC11 and _C9 libraries, see Example 2 for detailed description). Moreover, there is a clear enrichment in sequences derived from the "groove library" (scLib013_L16_AC11) as twenty out of the thirty PTH peptide-specific clones and all the sixteen PTHrP-specific clones are of this kind.

Clear sequence trends can be deduced from the alignments presented in Figures 5 and 6; notably:

Whereas five partly homologous groove library scAB variants were retrieved in case of PTHrP, one particular groove library sequence is clearly over-represented (occurring 17-fold) amongst the PTH peptide-specific scABs.

Three different PTH peptide-specific "surface" scAB variants (derived from the scLib_013_C9 library) were recovered; two of them are relatively homologous.

For comparison purposes, about 25 weakly positive clones that displayed PTH/PTHrP cross-reactivity were also analyzed. The vast majority belonged to the "short" scAB class. No meaningful trend could be deduced from the sequences except that glycine and tryptophan residues were frequent. The latter points towards non-specific selection (stickiness) and suggests thereby that this short format of the scaffold is less suited for the targets explored here.

Structure-based rationalization

The obtained binders to PTH and PTHrP have thusfar not been tested as soluble constructs. Although the ELISA signals were quite strong and reproducible, there still exists a risk that they bind in a structurally nonspecific manner. Although this is unlikely as we have been able to find PTH-specific binders that are not cross-reactive to PTHrP and *vice-versa*, we decided to try to rationalize the obtained sequences in structural terms. If successful, this will facilitate the design of future maturation libraries. The randomization was conducted by examining the sequence coherence between different variants as well as by model building and interactive ligand docking.

Rationalization of scAB binding to PTH

Sequence analysis of Lib013_AC11 PTH-binders (Figure 5) readily showed some consistency patterns. For example, comparing the 1_C1 and 21_G6 sequences, it is seen that the first three library residues of the A-helix (N/D, N/S and V/V) and the last four of the C-helix (A/A, W/M, G/G and L/L) are identical or homologous. A lower degree of similarity was also observed when comparing the clones 21_G6 with PTH_2_D6: two identical (D/D and R/R) and one homologous (L/V) residues in the A-helix and two homologous in the C-helix (M/Y and L/M).

Further, comparison of the Lib013_C9 PTH-binders revealed a similar situation (Figure 5): the first library position is charged (R/R/D); the second is identical (L/L/L); the third is twice Val (E/V/V); then 3 hydrophobic (W/F/L); 3 aliphatic (V/V/L); position 109 has twice an unintended -and therefore significant- Leu; followed by 3 small polar (N/G/S); and then, in the last two library positions each time one hydrophobic and one negatively charged residue, albeit not at the same position.

We reasoned that the observed homologies between independently obtained sequences are unlikely to be coincidences. Therefore, 3-D models were built for binders 1_C1 (scLib_L16_AC11-type) and 7_D2 (scLib_L16_C9-type), starting from our alpha-body scAB013_L16 template structure, by mutating each residue with preferred torsion angles. A clear groove was observed in the 1_C1 model, whereas an alternating knob-hole pattern was observed at the surface of the C-helix of 7_D2.

Next, PTH and PTHrP were examined. Knowing that the region 10-30 in PTH and PTHrP have an alpha-helical propensity in solution, we checked the possibility that these fragments would be bound in a similar mode as observed in 6-helix bundles of many viral fusion proteins (e.g. HIV-1 Env, Sars coronavirus, RSV-F). There, the groove-binding helices are also characterized by a classical heptad pattern. And indeed, PTH shows a 2-heptad a/d-pattern, while PTHrP shows a 1.5 heptad repeat:

```
                  .....................a..d...a..d...
PTH :       SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF
PTHrP :     AVSEHQLLHDKGKSIQDLRRRFFLHHLIAEIHTA
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With these insights in mind, we docked PTH in a helical conformation. With only a few side-chain rotations, this worked very well for the model 1_C1. Similarly, we could nicely dock PTH on top of the C-helix in a binding mode as observed in antiparallel dimeric coiled coils. All dockings could be realized without steric overlap. The modeled PTH-1_C1

complex is dominated by a large hydrophobic knobs-into-holes interface, complemented with small polar interactions at the N- and C-terminal ends.

Rationalization of scAB binding to PTHrP

Following a similar procedure for rationalization of the PTHrP-binding sequences (Figure 6), the latter also showed consistency patterns, splitting up into two apparent clusters: (i) 68_A11_R5 and 69_B11_R5 and (ii) 53_E'8, 65_C9_R5 and 62_B2_R5. No attempts were made to structurally rationalize the first cluster yet but the second cluster consisting of three independent variants was studied in detail. Especially the almost complete sequence similarity observed within the C-helix (Figure 6, right-most sequences) was striking: hydrophobic-Asn-L/I-L/I-hydrophobic. In the A-helix (left-most sequences) we observed a lot more polar residues and a lower degree of conservation, but still some consistency can be seen: hydrophobic-E/E/(A)-S/T-β-branched-small polar-(variable). When building the model for 62_B2_R5, it became clear that Ser/Thr at position 16 and the strictly conserved Asn102 form a deep hydrophilic pocket. The dimensions of the pocket were also suited to contain the guanidinium group of Arg21 of PTHrP. Moreover, the Thr16-Oγ1 and Asn102-Oδ1 atoms were at a suitable distance (7.2 Å) to serve as H-acceptors for the Arg hydrogen atoms. However, in order to dock the Arg inside the hydrophylic pocket, the PTHrP helix needed to be oriented parallel with the alpha-body groove helices (as opposed to the modeled PTH complexes). Yet, this was not considered improbable because similar orientations are observed in 4-helix bundles. Moreover, this orientation allowed all three aliphatic PTHrP a/d-positions to dock in a knobs-into-holes fashion into the hydrophobic pockets located at the C-terminal half of the alpha-body. So, this binding mode was deemed realistic for it could be generated without any steric overlap, with full shape complementarity and with both a hydrophobic and hydrophilic match.

CLAIMS

1. A single-chain protein comprising a PTHrP-binding coiled coil domain.

2. A PTHrP-binding single-chain coiled coil having a single contiguous amino acid chain with the formula HRS1-L1-HRS2-L2-HRS3, optionally supplemented with N- and C-terminal extensions resulting in the formula N-HRS1-L1-HRS2-L2-HRS3-C, wherein

a) each of HRS1, HRS2 and HRS3 is independently a heptad repeat sequence consisting of 2 to 7 consecutive heptad repeat units, at least 50% of all heptad a- and d-positions are occupied by isoleucine residues, and HRS1, HRS2 and HRS3 together constitute a 3-stranded alpha-helical coiled-coil structure;

b) each of L1 and L2 are independently a linker fragment, covalently connecting HRS1 to HRS2 and HRS2 to HRS3, respectively, starting and ending with a proline or glycine, and consisting of 3 to 30 amino acid residues of which at least 50% are selected from the group proline, glycine, serine;

c) N and C are each independently an optional extension, covalently connected to the N- and C-terminal ends of HRS1 and HRS3, respectively, these connections being marked by a helix-breaking proline or glycine.

3. The single-chain coiled coil of any of claims 1 or 2 which binds to the N-terminal part of human PTHrP, said N-terminal part being defined as amino acid residues 1 to 34 of SEQ ID NO: 1.

4. The single-chain coiled coil of any of claims 1 or 2 which binds to the midregion part of human PTHrP, said midregion

part being defined as amino acid residues 38 to 94 of SEQ ID NO: 1.

5. The single-chain coiled coil of any of claims 1 or 2 which binds to the nuclear localization sequence of human PTHrP, said sequence being defined as amino acid residues 88 to 106 of SEQ ID NO: 1.

6. The single-chain coiled coil of any of claims 1 or 2 which binds to the C-terminal part of the human PTHrP 1-139 isoform, said C-terminal part being defined as amino acid residues 107 to 139 of SEQ ID NO: 1.

7. The single-chain coiled coil of any of claims 1 or 2 which binds to the C-terminal part of the human PTHrP 1-173 isoform, said C-terminal part being defined as amino acid residues 140 to 173 of SEQ ID NO: 3.

8. The single-chain coiled coil of any of claims 1 or 2 which binds simultaneously to any pair of PTHrP parts from the group of PTHrP parts defined as amino acid residues 1 to 34, 38 to 94, 88 to 106, 107 to 139 and 140 to 173 of SEQ ID NO: 3, respectively.

9. The single-chain coiled coil of any of claims 1 or 2 wherein the binding to PTHrP is characterized by a dissociation constant (Kd) or half maximal effective concentration (EC50) in the submicromolar range, or the subnanomolar range, or the subpicomolar range.

10. The single-chain coiled coil of any of claims 1 or 2 wherein the binding to PTHrP inhibits the latter's binding to the PTHR1 receptor, characterized by a half maximal inhibitory concentration (IC50) in the submicromolar range, or the subnanomolar range, or the subpicomolar range.

11. The single-chain coiled coil of any of claims 1 or 2 wherein the binding to PTHrP inhibits the latter's signalling via the PTHR1 receptor, characterized by a half maximal

inhibitory concentration (IC50) in the submicromolar range, or the subnanomolar range, or the subpicomolar range.

12. The single-chain coiled coil of any of claims 1 or 2 wherein the binding to PTHrP inhibits the anti-apoptotic effect of PTHrP, characterized by a half maximal inhibitory concentration (IC50) in the submicromolar range, or the subnanomolar range, or the subpicomolar range.

13. The single-chain coiled coil of claim 12 wherein administration of said coiled coil to tumor cells inhibits tumor cell proliferation or induces tumor regression, and wherein said administration occurs in the submicromolar range, or the subnanomolar range, or the subpicomolar range.

14. A method for inhibiting tumor growth or inducing tumor regression in a patient in need thereof, comprising administering to the patient a PTHrP-binding single-chain coiled coil according to claim 13.

15. Use of the PTHrP-binding single-chain coiled coil according to any of claims 1 to 14 in the manufacture of a medicament for the treatment of disease.

16. A pharmaceutical composition comprising a PTHrP-binding single-chain coiled coil according to any of claims 1 to 13 and a pharmaceutically acceptable carrier.

17. A PTHrP-binding alpha-body according to claims 1 to 13 for use in the treatment or prevention of cancer, hypercalcemia associated with cancer, metastasis and/or tissue invasion.

18. A PTHrP-binding alpha-body according to claims 1 to 13 for use in the diagnosis of cancer, hypercalcemia associated with cancer, metastasis and/or tissue invasion.

19. A diagnostic agent comprising a PTHrP-binding alpha-body according to claims 1 to 13.

20. A nucleic acid molecule encoding an amino acid sequence of a PTHrP-binding single-chain coiled coil according to any of claims 1 to 8.

21. A host cell comprising a nucleic acid molecule according to claim 20.

Application No:	GB0911892.8	**Examiner:**	Richard Sewards
Claims searched:	1-21	**Date of search:**	30 October 2009

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
A	-	WO 2009/030780 A (COMPLIX) see p 2 l 30 - p 3 l 26, p 4 ll 25-30, p 6 l 24 - p 7 l 16, p 13 ll 7-35, Example 1
A	-	Journal of Molecular Biology (1998); Vol 281, pp 379-391, "From synthetic coiled coils...", Ghirlanda et al See whole document, esp. Abstract
A	-	EP 1283057 A (CHUGAI SEIYAKU KK) see paras 4-6 & 137-141
A	-	WO 97/04312 A (ICN PHARMACEUTICALS) see p 3 l 15 - p 4 l 12, p 22 l 7 - p 24 l 2

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC[X] :

Worldwide search of patent documents classified in the following areas of the IPC

C07K

The following online and other databases have been used in the preparation of this search report

WPI, EPODOC, TXTE, BIOSIS, MEDLINE

International Classification:

Subclass	Subgroup	Valid From
C07K	0014/00	01/01/2006
A61K	0038/16	01/01/2006
A61K	0038/29	01/01/2006
A61P	0035/00	01/01/2006

Subclass	Subgroup	Valid From
A61P	0035/04	01/01/2006
C07K	0014/635	01/01/2006