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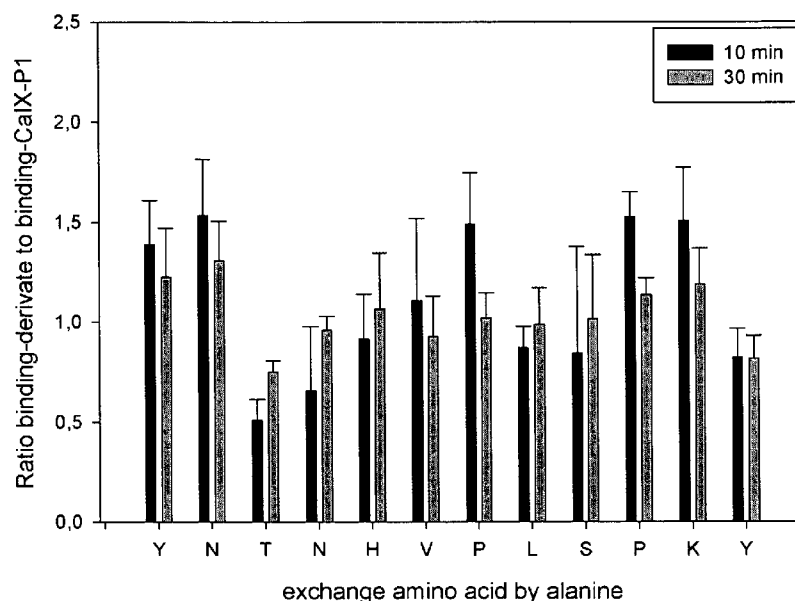
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(54) Title: TUMOUR TARGETING WITH POLYPEPTIDES

Figure 11

Alanine scanning of CalX-P1



(57) Abstract: The present invention relates to polypeptides which can be used to target tumours by binding to carbonic anhydrase IX (Ca9), in particular for diagnosis, prognosis and/or treatment of tumours. Further, it relates to methods of identifying such polypeptides.



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TUMOUR TARGETING WITH POLYPEPTIDES

The present invention relates to polypeptides which can be used to target tumours, in particular for diagnosis, prognosis and/or treatment of tumours. Further, it relates to methods of identifying such polypeptides.

BACKGROUND OF THE INVENTION

The outcome of cancer treatment can be influenced by the microenvironment within a solid tumour. One of the key factors for malignant tumour aggression and progression is hypoxia, representing an independent negative prognostic factor for therapy outcome. Tumour hypoxia is associated with a malign phenotype, characterized by high invasivity, increased potential for metastasis and a poor prognosis, resulting in reduced overall survival (Demir R et al., *Pathol Oncol Res.* 2009;15:417-22, Swinson DE et al., *J Clin Oncol.* 2003;21:473-82). Various experimental and clinical studies have confirmed the major role of hypoxia in treatment failure of both radiation therapy and chemotherapy (Rofstad EK et al., *Br J Cancer.* 2000;83:354-9). In particular, for more than 50 years it is known that subphysiologic levels of oxygen in the tumour lead to an up to 3-fold resistance to antineoplastic strategies such as radiation therapy compared to normoxic conditions (Bussink J et al., *Radiother Oncol.* 2003;67:3-15). The enhanced radioresistance is explained through various mechanisms. Oxygen deficiency leads to a reduced production of cytotoxic reactive species and promotes the upregulation of genes, like the vascular endothelial growth factor (VEGF), which not only induces angiogenesis but also protects the endothelial cells from irradiation (Jubb AM et al., *J Clin Pathol.* 2004;57:504-12). Furthermore, it is known that tumour hypoxia influences the migration activity of endothelial cells, resulting in an amplified signalling for angiogenesis (Giatromanolaki A et al., *Melanoma Res.* 2003;13:493-501). These facts show the necessity for the development of hypoxia imaging assays. In recent years some agents have been developed for imaging hypoxia using positron emission tomography (PET) (Imam SK (2010), *Cancer Biother Radiopharm* 25: 365-374). Among them fluorine-18-labeled fluoromisonidazole (^{18}F -FMISO) was thoroughly evaluated preclinical and in clinical trials and showed a significant higher uptake in hypoxic as in normoxic tumors. Furthermore, studies investigating ^{18}F -FMISO for hypoxia visualization demonstrated a correlation between tracer uptake and treatment response (Zimny M et al. (2006), *Eur J Nucl Med Mol Imaging* 33: 1426-1431; Thorwarth D et al. (2006), *Radiother Oncol* 80: 151-156).

On the cellular level, low oxygen tension results in the activation of a series of transcriptional regulators. One of them is hypoxia inducible factor 1 (HIF-1) (Semenza GL, *Trends Mol Med.* 2002; 8(suppl 4):62-7). HIF-1 is considered to have a central role as oxygen threshold in mammalian cells. Under hypoxic conditions, HIF-1 binds to hypoxia response elements (HRE) and induces the expression of hypoxia-response genes (Harris AL, *Nat Rev Cancer.* 2002;2:38-47). One of the inducible targets of HIF-1 transcriptional activity is carbonic anhydrase IX (CaIX) (Wykoff CC et al., *Cancer Res.* 2000;60:7075-83), which, therefore, can be used as an endogenous marker for cellular hypoxia.

Carbonic anhydrase IX is a member of a family of zinc metalloenzymes, which catalyse the hydration of carbon dioxide into carbonic acid. CaIX is a membrane associated glycoprotein, consisting of an extracellular catalytic domain extended with a proteoglycan-like region, which makes it easily accessible for targeting purposes, a transmembrane anchor and a short C-terminal cytoplasmic tail (Winum JY et al., *Med Res Rev.* 2008; 28:445-63). CaIX further is a tumour-associated member of the family of carbonic anhydrases that contributes to the acidification of extracellular pH and neutralization of intracellular pH protecting tumour cells from acidic pericellular microenvironment (Hulikova A et al., *FEBS Lett.* 2009;583:3563-8). CA IX as a tumor-associated antigen is linked to development of cancer in human beings (Pastorekova S et al. (1992), *Virology* 187: 620-626; Zavada J et al. (1993), *Int J Cancer* 54: 268-274; Liao SY et al. (1994), *Am J Pathol* 145: 598-609; Saarnio J et al. (1998), *Am J Pathol* 153: 279-285). The distribution of CA IX in human tissues exhibits a unique pattern that enables designation of CA IX as a tumor-associated protein (Thiry A et al. (2006), *Trends Pharmacol Sci* 27: 566-573).

The protein is not only found to be overexpressed in various human tumours, such as carcinomas of the colon, lung, ovaries, cervix and particularly the kidney (Niemelä AM et al., *Cancer Epidemiol Biomarkers Prev.* 2007;16:1760-6, McGuire BB and Fitzpatrick JM, *Curr Opin Urol.* 2009;19:441-6, Kim SJ et al., *Lung cancer.* 2005;49:325-35), but also various clinical studies have demonstrated a correlation between expression of CaIX and disease prognosis (Haapasalo JA et al., *Clin Cancer Res.* 2006;12:473-7, Skrzypski M et al., *Clin Cancer Res.* 2008;14:4794-9), indicating the predictive potential. In normal tissues CA IX expression is restricted to the gastrointestinal tract (Wykoff CC et al. (2000), *Cancer Res* 60: 7075-7083; Potter CP, Harris AL (2003), *Br J Cancer* 89: 2-7).

The leading role of tumour hypoxia in increased tumour resistance reveals the necessity for the development of hypoxia imaging assays. Such assays would allow a better characterization of tumour heterogeneity in respect of oxygenation, which is important for

planning, developing and carrying out targeted therapies such as radiation therapy, and the development of strategies for predicting treatment outcome. In this direction, nitroimidazole compounds find wide application. These compounds are reduced by intracellular reductases and subsequently bind to thiol groups of intracellular proteins, resulting in accumulation within hypoxic cells. A promising tool for detecting hypoxic regions within solid tumours is positron emission tomography (PET). In the past years several tracers have been developed for hypoxia imaging (Krause BJ et al., *Q J Nucl Med Mol Imaging*. 2006;50:28-43). Through labelling with radionuclides, nitroimidazole compounds find clinical application as PET tracers for measurement and imaging of tumour hypoxia (Mees G et al., *Eur J Nucl Med Mol Imaging*. 2009;36:1674-86). Fluorine-18-labeled fluoromisonidazole (^{18}F -FMISO) has been extensively evaluated in both preclinical and clinical trials demonstrating a significantly higher retention of the tracer in hypoxic than in normoxic tumours and a correlation between uptake and treatment response (Zimny M et al., *Eur J Nucl Med Mol Imaging*. 2006;33:1426-31, Thorwarth D et al., *Radiother Oncol*. 2006;80:151-6).

Still, there is increasing interest in the development of molecular imaging strategies based on ligands that bind selectively to target proteins that are overexpressed at hypoxic sites. Most common agents used for targeting purposes are antibodies. Monoclonal antibodies with high affinity to human carbonic anhydrase IX have already been generated and tested for diagnosis as well as for therapy (Chrastina A et al., *Int J Cancer*. 2003;105:873-81, Ahlskog JK et al., *Br J Cancer*. 2009;101:645-57, Brouwers AH et al., *Clin Cancer Res*. 2005;11:7178-7186.). Recently, monoclonal antibodies targeting CAIX have been developed and tested in preclinical and clinical trials. Investigation of the monoclonal antibody WX-G250 in phase III clinical trials indicated that the antibody is an attractive candidate for visualization and treatment of CAIX overexpressing clear cell renal cell carcinoma (Li G et al. (2007), *Clin Exp Metastasis* 24: 149-155; Li G et al. (2010), *Urol Oncol.*; Siebels M et al. (2011), *World J Urol* 29: 121-126). Further molecules with affinity for human carbonic anhydrase IX are sulfonamides. Sulfonamides can bind to CAIX, since they interact with the active center of the enzyme. A major drawback however is that sulfonamide binding on CAIX is characterized by low specificity since different members of the carbonic anhydrase family show high homology of their active centers. Studies have demonstrated that almost all sulfonamides bind to CA II, a molecule that is overexpressed in human erythrocytes. Use of sulfonamides for imaging purposes would lead to increased background values, which is disadvantageous for imaging purposes (Supuran CT WJ-Y (2009), Wang B, editor. Hoboken, New Jersey: John Wiley & Sons, Inc.).

An attractive alternative are peptides, i.e. short polypeptides. Peptides possess favourable pharmacokinetic properties through their small size, such as rapid clearance from blood, while they lack the immunogenic potential of antibodies. Furthermore, peptides are easy and cheap to synthesize. Therefore, there is increasing interest in the development of new peptide ligands with specific targeting abilities. However, the transfer of a new peptide to clinical applications can be difficult. A major drawback is the metabolic instability, which results in serum degradation, decreased tumor to organ ratios and enhanced background activity.

The inventors have developed a novel assay for the isolation of peptides which bind to CaIX. This method has been used to isolate a new peptide designated CaIX-Pep1, which can be used to target tumours for imaging, diagnosis, prognosis and/or treatment purposes. This new peptide has been analysed in detail to identify even shorter and more effective derivatives.

SUMMARY OF THE INVENTION

In a first aspect, the present invention relates to a polypeptide comprising an amino acid sequence according to SEQ ID NO: 1 or a variant thereof, wherein the variant has, with respect to SEQ ID NO: 1, up to 6 amino acid deletions and/or substitutions, preferably conservative substitutions

In a second aspect, the present invention relates to a method for isolating polypeptides which bind to a protein comprising the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and/or SEQ ID NO: 6 or a variant thereof, wherein a variant comprises an amino acid sequence which is at least 80% identical to the amino acid sequence according to one or more of said SEQ ID NOs, comprising the steps of:

- (i) contacting said protein with a library of candidate polypeptides,
- (ii) separating unbound polypeptides from said protein, and
- (iii) optionally eluting bound polypeptides from said protein and optionally repeating steps (i) to (iii) after enriching the eluted polypeptides,
- (iv) identifying the remaining polypeptides bound to or eluted from said protein and optionally repeating steps (i) to (iv).

In a third aspect, the present invention relates to a method of targeting a cell expressing a protein comprising the amino acid sequence according to SEQ ID NO: 2, 3, 4, 5 and/or 6 or a variant thereof, wherein a variant comprises an amino acid sequence which is at least 80% identical to the amino acid sequence according to one or more of said SEQ ID NOs,

using a polypeptide according to the invention or a polypeptide isolated with the method according to the invention.

In a fourth aspect, the present invention relates to the use of a polypeptide according to the invention or a polypeptide isolated with the method according to the invention in
5 diagnosis, prognosis and/or treatment of a tumour.

This summary of the invention does not necessarily describe all features of the invention.

DETAILED DESCRIPTION OF THE INVENTION

10 Before the present invention is described in detail below, it is to be understood that this invention is not limited to the particular methodology, protocols and reagents described herein as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims. Unless defined
15 otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art.

Preferably, the terms used herein are defined as described in "A multilingual glossary of biotechnological terms: (IUPAC Recommendations)", Leuenberger, H.G.W, Nagel, B. and Kölbl, H. eds. (1995), Helvetica Chimica Acta, CH-4010 Basel, Switzerland).

20 Several documents are cited throughout the text of this specification. Each of the documents cited herein (including all patents, patent applications, scientific publications, manufacturer's specifications, instructions, GenBank Accession Number sequence submissions etc.), whether supra or infra, is hereby incorporated by reference in its entirety. Nothing herein is to be construed as an admission that the invention is not entitled to antedate
25 such disclosure by virtue of prior invention.

In the following, the elements of the present invention will be described. These elements are listed with specific embodiments, however, it should be understood that they may be combined in any manner and in any number to create additional embodiments. The variously described examples and preferred embodiments should not be construed to limit the
30 present invention to only the explicitly described embodiments. This description should be understood to support and encompass embodiments which combine the explicitly described embodiments with any number of the disclosed and/or preferred elements. Furthermore, any permutations and combinations of all described elements in this application should be

considered disclosed by the description of the present application unless the context indicates otherwise.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integer or step.

As used in this specification and the appended claims, the singular forms “a”, “an”, and “the” include plural referents, unless the content clearly dictates otherwise.

The present invention relates to a polypeptide comprising, consisting essentially of or consisting of an amino acid sequence $Y_1N_2T_3N_4H_5V_6P_7L_8S_9P_{10}K_{11}Y_{12}$ (SEQ ID NO: 1, the numericals are merely provided for easier reference to the individual amino acids) or a variant thereof, wherein the variant has, with respect to SEQ ID NO: 1, 1, up to 2, up to 3, up to 4, up to 5 or up to 6 amino acid deletions or substitutions. 1, 1, up to 2, up to 3, up to 4, up to 5 or up to 6 amino acid deletions and substitutions.

Generally, the amino acids of SEQ ID NO: 1 can be substituted with any amino acid. In a preferred embodiment, the substitutions are conservative. The term “conservative amino acid substitution” refers to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine and tryptophan; a group of amino acids having basic side chains is lysine, arginine and histidine. Preferred conservative amino acid substitutions are: valine-isoleucine-leucine; tyrosine-phenylalanine; lysine-arginine; alanine-valine and asparagine-glutamine. In a preferred embodiment, the polypeptides of the invention have an amino acid sequence according to the following formula (I): $Y-X_1-X_2-X_3-H-X_4-P-L-X_5-P-X_6-Y$ (SEQ ID NO: 7), wherein X_1 is N or Q, X_2 is T or S, X_3 is N or Q, X_4 is V, L or I, X_5 is S or T and X_6 is K or R. In this context it is also envisioned in a preferred embodiment that the 1, 2, 3 or 4 amino acids are deleted N-terminally and 1, 2 or 3 amino acids are deleted C-terminally, i.e. the following deletion combinations (C-terminally and N-terminally): 1/0, 2/0, 3/0, 4/0, 1/1, 2/1, 3/1, 4/1, 1/2, 2/2, 3/2, 4/2, 1/3, 2/3, 3/ or 4/3.

In another embodiment, the substitutions of any amino acid of SEQ ID NO: 1 can be with any unnatural amino acid known in the art. The term “unnatural amino acids” refers to non-genetically-coded amino acids that either occur naturally or are chemically synthesised.

Groups of unnatural amino acids are, for example, α -amino acids, β -amino acids (β^3 and β^2), homo-amino acids, cyclic amino acids, aromatic amino acids, Pro and Pyr derivatives, 3-substituted Alanine derivatives, Glycine derivatives, ring-substituted Phe and Tyr Derivatives, Linear Core Amino Acids, N-methylated amino acids Diamino acids and D-amino acids. In a preferred embodiment the amino acid sequence of the variants only differ with respect to the amino acid sequence according to SEQ ID NO: 1 at one of positions X_1 , X_2 , X_3 , X_4 , X_5 or X_6 or at two positions selected from X_1 and X_2 , X_1 and X_3 , X_1 and X_4 , X_1 and X_5 , X_1 and X_6 , X_2 and X_3 , X_2 and X_4 , X_2 and X_5 , X_2 and X_6 , X_3 and X_4 , X_3 and X_5 , X_3 and X_6 , X_4 and X_5 , X_4 and X_6 and X_5 and X_6 . It is also known that one or more amino acids can be replaced by structural mimetics, e.g. taurine can replace Ala and pyridine can substitute Ala-Pro. Such mimetics of amino acids are used in the art to prolong the half life of polypeptides, e.g. to render them more protease resistant.

In one embodiment, one or more amino acids of the polypeptides of the invention are D-amino acids, i.e. 1, up to 2, up to 3, up to 4, up to 5, up to 6, up to 7, up to 8, up to 9, up to 10, up to 11, up to 12.

The polypeptides of the invention can also have up to 6, up to 5, up to 4, up to 3, up to 2 or 1 amino acid deletions. The polypeptide comprising deletions can have 1, up to 2, up to 3, up to 4, up to 5 or up to 6 amino acid substitutions, preferably conservative amino acid substitutions. For example, the polypeptide can have up to 3 deletions and up to 1, 2 or 3 substitutions, up to 2 deletions and up to 1, 2 or 3 substitutions, or 1 deletion and up to 1, 2 or 3 substitutions.

In one embodiment, the sequence variant of said polypeptide has up to 6, i.e. 0, 1, 2, 3, 4, 5 or 6 amino acid deletions. In another embodiment, the sequence variant of said polypeptide has up to 5, i.e. 0, 1, 2, 3, 4 or 5 amino acid deletions and optionally one, i.e. 0 or 1 amino acid substitution, preferably a conservative amino acid substitution. In yet another embodiment, the sequence variant of said polypeptide has up to 4, i.e. 0, 1, 2, 3 or 4 amino acid deletions and optionally up to two, i.e. 0, 1 or 2 amino acid substitutions, preferably conservative amino acid substitutions.

It is preferred that said deletions are terminal deletions, i.e. amino acids at the N-terminal and/or C-terminal end of the sequence are preferably deleted.

In one preferred embodiment, said sequence variant has a length of between 6 to 12, preferably 6 to 11, 6 to 10, 6 to 9, 6 to 8, 6 to 7 amino acids, i.e. up to 3, i.e. 0, 1, 2 or 3 N-terminal amino acid deletions and up to 3, i.e. 0, 1, 2 or 3 C-terminal amino acid deletions, up to 4, i.e. 0, 1, 2, 3 or 4 N-terminal amino acid deletions and up to 2, i.e. 0, 1 or 2 C-terminal

amino acid deletions, or up to 2, i.e. 0, 1 or 2 N-terminal amino acid deletions and up to 4, i.e. 0, 1, 2, 3 or 4 C-terminal amino acid deletions. Thus, preferably the amino acid span positions 1 to 11, 1 to 10, 1 to 9, 2 to 12, 3 to 12, 4 to 12, 5 to 12, 2 to 11, 2 to 10, 2 to 9, 3 to 11, 3 to 10, 3 to 9, 4 to 11, 4 to 10, or 4 to 9 (the positions are indicated on the basis of the numbering set out in Y₁N₂T₃N₄H₅V₆P₇L₈S₉P₁₀K₁₁Y₁₂). In this context substitutions, preferably conservative substitutions are allowable. Preferably, the sequence variant has a sequence according to SEQ ID NO: 8 (N₄H₅V₆P₇L₈S₉), SEQ ID NO: 9 (T₃N₄H₅V₆P₇L₈), SEQ ID NO: 10 (H₅V₆P₇L₈S₉P₁₀), SEQ ID NO: 11 (H₅V₆P₇L₈S₉P₁₀K₁₁), SEQ ID NO: 12 (N₄H₅V₆P₇L₈S₉P₁₀), SEQ ID NO: 13 (T₃N₄H₅V₆P₇L₈S₉), SEQ ID NO: 14 (N₂T₃N₄H₅V₆P₇L₈), SEQ ID NO: 15 (T₃N₄H₅V₆P₇L₈S₉P₁₀), SEQ ID NO: 16 (N₂T₃N₄H₅V₆P₇L₈S₉), or SEQ ID NO: 17 (N₄H₅V₆P₇L₈S₉P₁₀K₁₁).

In another preferred embodiment, said sequence variant has up to 4, i.e. 0, 1, 2, 3 or 4 N-terminal amino acid deletions and up to 1, i.e. 0 or 1 C-terminal amino acid deletion, up to 3, i.e. 0, 1, 2 or 3 N-terminal amino acid deletions and up to 2, i.e. 0, 1 or 2 C-terminal amino acid deletions, up to 2, i.e. 0, 1 or 2 N-terminal amino acid deletions and up to 3, i.e. 0, 1, 2 or 3 C-terminal amino acid deletions, or up to 1, i.e. 0 or 1 N-terminal amino acid deletion and up to 4, i.e. 0, 1, 2, 3 or 4 C-terminal amino acid deletions, and wherein, if applicable, said sequence variant optionally has 1, i.e. 0 or 1 non-terminal deletion, and wherein, preferably, the sequence variant has a sequence according to SEQ ID NO: 18 (H₅V₆P₇L₈S₉P₁₀K₁₁), SEQ ID NO: 19 (N₄H₅V₆P₇L₈S₉P₁₀), SEQ ID NO: 20 (T₃N₄H₅V₆P₇L₈S₉), SEQ ID NO: 21 (N₂T₃N₄H₅V₆P₇L₈), SEQ ID NO: 22 (T₃N₄H₅V₆P₇L₈S₉P₁₀), SEQ ID NO: 23 (N₂T₃N₄H₅V₆P₇L₈S₉), or SEQ ID NO: 24 (N₄H₅V₆P₇L₈S₉P₁₀K₁₁).

In yet another preferred embodiment, said sequence variant has up to 2, i.e. 0, 1 or 2 N-terminal amino acid deletions and up to 2, i.e. 0, 1 or 2 C-terminal amino acid deletions, up to 1, i.e. 0 or 1 N-terminal amino acid deletion and up to 3, i.e. 0, 1, 2, or 3 C-terminal amino acid deletions, or up to 3, i.e. 0, 1, 2, or 3 N-terminal amino acid deletions and up to 1, i.e. 0 or 1 C-terminal amino acid deletion, and wherein, if applicable, said sequence variant optionally has 1, i.e. 0 or 1 non-terminal deletion, and wherein, preferably, the sequence variant has a sequence according to SEQ ID NO: 25 (T₃N₄H₅V₆P₇L₈S₉P₁₀), SEQ ID NO: 26 (N₂T₃N₄H₅V₆P₇L₈S₉), or SEQ ID NO: 27 (N₄H₅V₆P₇L₈S₉P₁₀K₁₁). In general and in particular in all embodiments described above or below, it is preferred that the amino acids H₅V₆ or V₆P₇ or preferably H₅V₆P₇ (SEQ ID NO: 28) and the amino acids N₄ or L₈, preferably L₈S₉ or more preferably L₈S₉P₁₀ are not deleted. In other words, the sequence variant of the invention

comprises the amino acids H₅V₆ or V₆P₇ or preferably H₅V₆P₇ and the amino acids N₄ or L₈, preferably L₈S₉ or more preferably L₈S₉P₁₀ (SEQ ID NO: 29).

It is apparent from the examples that, surprisingly, also polypeptides can be used for the purpose of the invention which comprise a sequence variant of SEQ ID NO: 1 containing
5 more than 6 deletions and/or amino acid substitutions, preferably conservative substitutions. For example, the invention also relates to a polypeptide comprising an amino acid sequence according to SEQ ID NO: 1 (Y₁N₂T₃N₄H₅V₆P₇L₈S₉P₁₀K₁₁Y₁₂) or a sequence variant thereof, wherein said sequence variant has, with respect to SEQ ID NO: 1, 7 or 8 terminal amino acid deletions, wherein said sequence variant comprises the amino acid V₆ of SEQ ID NO: 1 and
10 said sequence variant optionally comprises one substitution, preferably conservative substitution, wherein preferably said polypeptide is capable of binding under physiological conditions to a protein comprising the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 3 SEQ ID NO: 4, SEQ ID NO: 5 and/or SEQ ID NO: 6 or a variant thereof, wherein said variant comprises an amino acid sequence which is at least 80%, at least 85%, at least
15 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the amino acid sequence according to one or more of said SEQ ID NOs. In a preferred embodiment, this sequence variant comprises, essentially consists of or consists of the amino acids H₅V₆ or V₆P₇ of SEQ ID NO: 1, preferably the amino acids N₄H₅V₆, (SEQ ID NO: 30), H₅V₆P₇ (SEQ ID NO: 28) or V₆P₇L₈ (SEQ ID NO: 31) of SEQ ID NO: 1, more preferably the
20 amino acids T₃N₄H₅V₆ (SEQ ID NO: 32), N₄H₅V₆P₇ (SEQ ID NO: 33), H₅V₆P₇L₈ (SEQ ID NO: 34) or V₆P₇L₈S₉ (SEQ ID NO: 35) of SEQ ID NO: 1 or the amino acids N₂T₃N₄H₅V₆ (SEQ ID NO: 36), T₃N₄H₅V₆P₇ (SEQ ID NO: 37), N₄H₅V₆P₇L₈ (SEQ ID NO: 38), H₅V₆P₇L₈S₉ (SEQ ID NO: 39) or V₆P₇L₈S₉P₁₀ (SEQ ID NO: 40) of SEQ ID NO: 1. These peptides comprising shorter stretches of the peptide according to SEQ ID NO: 1 can be used
25 also in the context of all the other embodiments and preferred embodiments described above and below with respect to the derivatives of SEQ ID NO: 1 with up to 6 substitutions and/or deletions.

In a preferred embodiment of the short polypeptides of the present invention the polypeptide has a length of between 3 to 30 amino acids, preferably of 3, 4, 5, 6, 7, 8, 9, 10,
30 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids and comprises N₄H₅V₆. In a further preferred embodiment of the short polypeptides of the present invention the polypeptide has a length of between 3 to 30 amino acids, preferably of 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids and comprises H₅V₆P₇. In a further preferred embodiment of the short polypeptides of the present invention the polypeptide has a length of between 3 to 30

amino acids, preferably of 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids and comprises $V_6P_7L_8$. In a further preferred embodiment of the short polypeptides of the present invention the polypeptide has a length of between 4 to 30 amino acids, preferably of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids and comprises

5 $T_3N_4H_5V_6$. In a further preferred embodiment of the short polypeptides of the present invention the polypeptide has a length of between 4 to 30 amino acids, preferably of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids and comprises $N_4H_5V_6P_7$. In a further preferred embodiment of the short polypeptides of the present invention the polypeptide has a length of between 4 to 30 amino acids, preferably of 4, 5, 6, 7, 8, 9, 10, 11,

10 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids and comprises $H_5V_6P_7L_8$. In a further preferred embodiment of the short polypeptides of the present invention the polypeptide has a length of between 4 to 30 amino acids, preferably of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids and comprises $V_6P_7L_8S_9$. In a further preferred embodiment of the short polypeptides of the present invention the polypeptide has a length of between 5 to 30

15 amino acids, preferably of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids and comprises $N_2T_3N_4H_5V_6$. In a further preferred embodiment of the short polypeptides of the present invention the polypeptide has a length of between 5 to 30 amino acids, preferably of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids and comprises $T_3N_4H_5V_6P_7$. In a further preferred embodiment of the short polypeptides of the present

20 invention the polypeptide has a length of between 5 to 30 amino acids, preferably of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids and comprises $N_4H_5V_6P_7L_8$. In a further preferred embodiment of the short polypeptides of the present invention the polypeptide has a length of between 5 to 30 amino acids, preferably of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids and comprises $H_5V_6P_7L_8S_9$. In a further preferred

25 embodiment of the short polypeptides of the present invention the polypeptide has a length of between 5 to 30 amino acids, preferably of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids and comprises $V_6P_7L_8S_9P_{10}$. In these embodiments it is also envisioned to substitute amino acids, preferably 1, 2, 3, or 4, preferably 1 or 2, more preferably 1 by non-natural amino acids, mimetics of amino acids or non-natural amino acids and mimetics of

30 amino acids, as set out above and wherein the resulting short polypeptide variant is capable of binding to, e.g. under physiological conditions, a protein comprising the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 3 SEQ ID NO: 4, SEQ ID NO: 5 and/or SEQ ID NO: 6 or a variant thereof, wherein said variant comprises an amino acid sequence which is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or

at least 99% identical to the amino acid sequence according to one or more of said SEQ ID NOs.

While it is envisaged that the sequence variant of the invention may comprise 6 substitutions and/or deletions, including terminal and/or non-terminal deletions, it is preferred that not more than 6, 5, 4, 3, preferably 2 or 1 amino acids in total are substituted, conservatively substituted and/or non-terminally deleted.

With respect to non-terminal deletions, it is preferred that said non-terminal deletions are selected from the group consisting of N₂/K₁₁, T₃/P₁₀, S₉, L₈, P₁₀, N₄, P₇, V₆, and H₅ in order of preference, wherein / indicates equal preference rather than the deletion of two amino acids. This order of preference reflects the activity of peptide derivatives containing non-terminal deletions according to Example 16, in particular Figure 17.

It is further preferred that said substitution and/or said conservative substitution concerns the amino acid selected from the group consisting of Y₁/N₂/T₃/K₁₁/Y₁₂, N₄, H₅, S₉, P₇, V₆, P₁₀, and L₈ in order of preference, wherein / indicates equal preference rather than the substitution of two amino acids. This order of preference reflects the activity of peptide derivatives containing non-terminal deletions according to Example 16, in particular Figure 16.

It is also envisaged that one or more of the amino acids of the polypeptide of the invention, in particular of the amino acids of SEQ ID NO: 1 are methylated, e.g. Y₁, N₂, T₃, N₄, H₅, V₆, P₇, L₈, S₉, P₁₀, K₁₁, and/or Y₁₂. In a preferred embodiment, the amino acid S₉ according to SEQ ID NO: 1 is methylated.

Generally it is preferred that the polypeptide of the invention is up to 1000, up to 500, up to 250, up to 100, preferably up to 50, more preferably up to 25, more preferably up to 15, and most preferably up to 12 amino acids long, and/or wherein said peptide is at least 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 amino acids long.

Furthermore, it is envisaged that in one aspect of the invention, the sequence variant of the invention does not have a sequence according to SEQ ID NO: 1 (Y₁N₂T₃N₄H₅V₆P₇L₈S₉P₁₀K₁₁Y₁₂), i.e. that a sequence according to SEQ ID NO: 1 is excluded from the scope of all embodiments described herein and from the appended claims.

As readily apparent from the method described herein which lead to the identification of CaIX-P1, it is preferred that the polypeptide of the invention is capable of binding, e.g. under physiological conditions to a protein comprising the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 3 SEQ ID NO: 4, SEQ ID NO: 5 and/or SEQ ID NO: 6 or a variant thereof, wherein said variant comprises an amino acid sequence which is at least 80%,

at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the amino acid sequence according to one or more of said SEQ ID NOs. Said binding preferably occurs at physiological conditions which refers to conditions of the external or internal milieu that occurs in nature for an organism, preferably an animal, more preferably a mammal and most preferably a human, or a cell system, in contrast to arbitrary laboratory conditions. Preferably, physiological conditions comprise one or more of the following factors: a temperature range of 20-40°C, atmospheric pressure of 80-120 kPa, preferably about 1 atm or 101,325 Pa, pH of 6-8, glucose concentration of 1-20 mM, atmospheric gas concentrations (preferably comprising one or more of 60-95% nitrogen, preferably about 78.08% nitrogen, 10-30 % oxygen, preferably about 20.95% oxygen, a variable amount, i.e. 0-10% water vapor, preferably around 1.247% water vapor, 0.1-10 % argon, preferably about 0.93% argon, 0.01-10% carbon dioxide, preferably 0.038% carbon dioxide, and optionally traces of hydrogen, helium, and other noble gases), and/or earth gravity (about 9.81 m/s²).

The skilled person can readily identify further functional sequence variants which are within the scope of the invention, without the need to test every theoretically possible sequence. Candidate peptides can be synthesised, for example by solid phase synthesis as described in Examples 3 and 14. The present application provides ample guidance on which amino acids are important for the peptide function according to the invention, particularly in Examples 14 and 16. Suitability, e.g. binding efficacy of candidate sequence variants can be assessed as described in the examples. The skilled person is well aware of appropriate assays, which include phage display (see Example 2), binding tests using immobilised CaIX (see Example 4), *in vitro* binding using radioligands (see Examples 5 and 15), competition tests (see Example 14), internalisation tests (see Example 15), binding specificity tests (see Example 21), stability tests (see Examples 11 and 19), visualisation of tumour targeting (see Example 12), or organ distribution assays (see Examples 13 and 18). In summary, in view of the teaching provided by the present application, the skilled person can purposively select further sequence variants not explicitly disclosed in this application and test their suitability, in particular the binding to CaIX and/or tumour targeting, e.g. the targeting of tumours described below in the section relating to "targeting", without undue burden.

In one embodiment of the invention, the polypeptides of the invention are labelled. The term "labelling" refers to a modification of said polypeptide using an atom or molecule which allows identification of said polypeptide. Examples are radioactive isotopes or tags.

Further, the polypeptides of the invention can be coupled directly or indirectly to one or more tags, chelators, imaging agents and/or therapeutic agents. For example, tags are selected from the group consisting of His-tag, oligo-aspartate-tag, tetracysteine-tag, and lanthanide-binding-tag, the chelators are selected from the group consisting of EDTA, NOTA, TETA, Iminodiacetic acid, DOTA, DTPA, and HYNIC, the imaging agents are selected from the group consisting of radioactive molecules and ions, paramagnetic ions, fluorogenic ions, chromophors, small fluorescent molecules, e.g. bioaresenical dyes, contrast enhancing agents, e.g. Gd, Eu or Mn containing molecules, chelators binding metals or ions of radionuclides detectable in imaging procedures, and the therapeutic agents are selected from the group consisting of radioactive molecules or isotopes, alkylating agents, anthracyclines, cytoskeletal disruptors, epothilones, inhibitors of topoisomerase II, nucleotide analogs and precursor analogs, peptide antibiotics, platinum-based agents, retinoids, vinca alkaloids and derivatives, and cytotoxic/cytostatic agents such as A chain toxin, ribosome inactivating proteins, α -sarcin, aspergillin, restrictotin, diphtheria toxin, Pseudomonas exotoxin, bacterial endotoxins or their lipid A moieties, Bleomycin, Dactinomycin, Mitomycin, Daunorubicin, Doxorubicin, Epirubicin, Idarubicin, Mitoxantron, Amascrin, Doxofluridin, Cisplatin, Carboplatin, Oxaliplatin, Satraplatin, Camptothecin, Toptecan, Irinotecan, Amsacrin, Etoposid, Teniposid, Cyclophosphamid, Ifosfamid, Trofosfamid, Melphalan, Chlorambucil, Estramustin, Busulfan, Chlorambucil, Chlormethin, Treosulfan, Carmustin, Lomustin, Nimustin, Streptozocin, Procarbazine, Streptozocin, Dacarbazine, Ifosfamid, Temozolomid, Thiotepa, Vinorelbin, Vincristin, Vinblastin, Vindesine, Paclitaxel, Docetaxel, Methotrexate, Pemetrexed, Raltitrexed, Fluorouracil, Capecitabine, Cytosine arabinoside, Gemcitabine, Tioguanine, Pentostatin, Azathioprine, Mercaptopurine, Fludarabine, Caldribrin, Hydroxycarbamide, Mitotane, Azacitidine, Cytarabine, Gemcitabine, Nelarabine, Bortezomib, Anagrelide and protein kinase inhibitors like Imatinib, Erlotinib, Sunitinib, Sorafenib, Dasatinib, Lapatinib or Nilotinib.

The term "direct coupling" refers to a direct covalent or non-covalent bond, preferably covalent bond between the polypeptide of the invention to one or more tags, chelators, imaging agents and/or therapeutic agents. The term "indirect coupling" is used to refer to the situation wherein a linker is positioned between the polypeptide of the invention and the one or more tags, chelators, imaging agents and/or therapeutic agents. The polypeptide is preferably coupled at the N- or C-terminus to the one or more tags, chelators, imaging agents and/or therapeutic agents. It is, however, also envisioned that the coupling is carried out via an internal amino acid, e.g. an amino acid with a reactive or activatable side chain like lysine,

arginine, glutamine, asparagine, serine or cysteine. Such coupling is carried out in a way, which essentially does not alter binding of the polypeptide to CaIX-P1.

Preferred chelators are those binding metal ions which can be detected with imaging methods such as SPECT, PET, CT or MRT. Examples for suitable metal ions are Fe^{2+} , Fe^{3+} , Cu^{2+} , Cr^{3+} , Gd^{3+} , Eu^{3+} , Dy^{3+} , La^{3+} , Yb^{3+} and/or Mn^{2+} or the ions of radionuclides such as gamma-emitters, positron-emitters, Auger-electron-emitters, alpha-emitters, X-ray-emitters and fluorescence-emitters, e.g. ^{51}Cr , ^{67}Ga , ^{68}Ga , ^{111}In , $^{99\text{m}}\text{Tc}$, ^{140}La , ^{175}Yb , ^{153}Sm , ^{166}Ho , ^{88}Y , ^{90}Y , ^{149}Pm , ^{177}Lu , ^{47}Sc , ^{142}Pr , ^{159}Gd , ^{212}Bi , ^{72}As , ^{72}Se , ^{97}Ru , ^{109}Pd , ^{105}Rh , $^{101\text{m}}\text{Rh}$, ^{119}Sb , ^{128}Ba , ^{197}Hg , ^{211}At , ^{169}Eu , ^{203}Pb , ^{212}Pb , ^{64}Cu , ^{67}Cu , ^{188}Re , ^{186}Re , ^{198}Au und/oder ^{199}Ag .
Examples for applications are ^{111}In for SPECT, ^{68}Ga for PET, ^{90}Y for therapy, Gd, Eu, Mn for MRT, Gadolinium, Wolfram or other elements with high atomic number for CT. Above examples are to be construed non-limiting and other tags, chelators, imaging agents and/or therapeutic agents known in the art can be used as well.

The polypeptides of the invention can be modified by any of the means of the group consisting of substituting one or more atoms with radioactive isotopes, cyclisation, acetylation, pegylation, N-methylation, protecting an N-terminal tyrosine with a t-butyloxycarbonyl group, and providing said polypeptide with a scaffold structure. Said providing can be achieved by fusing said polypeptide to a scaffold structure on DNA or protein level, by introducing substitutions or insertions to graft the sequence of said polypeptide onto the surface of a protein scaffold structure or by other means for providing scaffold structures as described below.

The present invention also relates to polynucleotides encoding for the polypeptides of the invention. The sequences of said polynucleotides can be derived from the sequence of said polypeptides according to the genetic code.

The present invention also relates to a vector comprising the polynucleotides of the invention. Such vectors can be cloning and expression vectors. The term "vector" relates to a DNA molecule used as a vehicle to clone, carry, transfer and/or express genetic material. Non-limiting examples for vectors are plasmids, viruses including bacteriophages, cosmids, and artificial chromosomes.

The present invention also relates to a method for isolating polypeptides which bind to a protein comprising the amino acid sequence according to SEQ ID NO: 2 (full-length human carbonic anhydrase IX), SEQ ID NO: 3 (extracellular domain of human carbonic anhydrase IX, amino acids 1-414 of human carbonic anhydrase IX), SEQ ID NO: 4 (part of the proteoglycan like region of human CaIX, amino acids 38-112 of human carbonic anhydrase

IX), SEQ ID NO: 5 (part of the proteoglycan like region of human CaIX, amino acids 53-112 of human carbonic anhydrase IX) and/or SEQ ID NO: 6 (catalytic domain of human CaIX, amino acids 113-414 of human carbonic anhydrase IX) or a variant thereof, wherein a variant comprises an amino acid sequence which is at least 80% identical to the amino acid sequence

5 according to one or more of said SEQ ID NOs, comprising the steps of:

- (i) contacting said protein with a library of candidate polypeptides,
- (ii) separating unbound polypeptides from said protein, and
- (iii) optionally eluting bound polypeptides from said protein and optionally repeating steps (i) to (iii) after enriching the eluted polypeptides,
- 10 (iv) identifying the remaining polypeptides bound to or eluted from said protein and optionally repeating steps (i) to (iv).

The term “isolating” refers to the identification of one or more polypeptides among a group of candidate polypeptides which is generally larger than the number of said one or more polypeptides. It is not to be construed as an isolation in terms of (bio)chemical
15 purification.

The term “variant” refers to a polypeptide which is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the amino acid sequence according to SEQ ID NOs 2, 3, 4, 5 and/or 6.

The term “library of candidate polypeptides” refers to a group of polypeptides
20 comprising one or more contiguous stretches of variable amino acid sequences, which are optionally embedded in an invariable consensus sequence and/or in a sequence providing a scaffold structure. The library of candidate polypeptides can be unbiased, wherein the variable amino acid sequence(s) is/are chosen randomly, or biased, wherein the variable amino acids sequence(s) is/are chosen based on prior knowledge, which can, for example, be based
25 information gained during previous repeats of steps (i) to (iv). It can also be partly biased, i.e. one or more variable amino acids are chosen randomly and one or more amino acids are chosen based on prior knowledge.

The term “contacting” refers to bringing said protein together with said candidate polypeptides so that binding between these entities is possible. In one embodiment, said
30 protein is immobilised, preferably on a surface, and said candidates polypeptides preferably are in solution which covers at least part of said surface. In other embodiments, both candidate polypeptides and protein are in solution or said polypeptides are immobilised, preferably on a surface, and said protein preferably is in solution covering at least part of said surface. Said protein can also be presented on a cell or contained in a cell *ex vivo* or *in vivo*.

The term “separating” refers to physically separating unbound polypeptides so that said protein cannot come into contact with said unbound polypeptides anymore. In a preferred embodiment, said immobilised protein or said immobilised candidate polypeptides are washed with a washing solution not containing any candidate polypeptides or said protein, respectively. If both protein and candidate polypeptides are present in a solution, one or the other can be immobilised prior to such a washing step.

The term “eluting bound polypeptides” refers to the disassociation of protein and bound polypeptides by increasing their dissociation constant, for example by changing temperature, pH, and/or salt concentration.

The term “enriching the eluted polypeptides” refers to an amplification or multiplication of the eluted polypeptides.

The term “identifying the remaining polypeptides” refers to determining the sequence of the amino acids of said polypeptides, either on amino acid or on nucleotide level.

In one embodiment, above method further comprises before step (i) a negative selection, comprising the following steps:

- (a) contacting said library of candidate polypeptides with a negative target protein or a domain thereof,
- (b) separating unbound polypeptides from said negative target protein or domain thereof, wherein said negative target protein or domain thereof does not comprise the amino acid sequence according to SEQ ID NO: 2, 3, 4, 5 or 6 or a variant thereof, wherein a variant comprises an amino acid sequence which is at least 80% identical to the amino acid sequence according to one or more of said SEQ ID NOs, wherein only the unbound polypeptides of step (b) are further processed in step (i) of above-described method for isolating polypeptides, and wherein steps (a) and (b) are optionally repeated with the optional repeats of steps (i) to (iii) or (i) to (iv).

Optionally two or more different negative target proteins are used, either one or more than one per repeat of steps (a) and (b).

In another embodiment, above method comprises before step (i) and/or before an optional negative selection a background negative selection, wherein candidate polypeptides are pre-adsorbed without any target protein, i.e. without a protein of the invention or a negative target protein, for example on a surface of the type on which target proteins are to be immobilised, and wherein only free, i.e. non-binding candidate polypeptides are used in further steps.

The term “negative target protein” refers to a protein which is substantially different from any of the proteins according to SEQ ID NO: 2, 3, 4, 5 and/or 6, wherein “substantially different” means that the amino acid sequence of the negative target protein is less than 10%, 20%, 30%, 40%, 60%, 70%, 80%, 85%, 90% or 95% identical to the amino acid sequence represented by SEQ ID NOs 2, 3, 4, 5 or 6 over a length of the latter. In one embodiment, said negative target protein is expressed, preferably overexpressed, in tumours. In another embodiment, said negative target protein is a receptor protein or comprises the extracellular domain of a receptor protein. In above-described method, one negative target protein or two or more different negative target proteins are used. Preferably, a negative target protein is a mammalian protein and even more preferably, a human protein.

Said candidate polypeptides each comprise 6-100, 6-90, 6-80, 6-70, 6-60, 6-55, 6-50, 6-45, 6-40, 6-35, 6-30, 6-25, 6-20, 6-18, 6-15, 6-12, 6-11, 6-10, 6-9, 6-8, 6-7, or 6 amino acids and at least one continuous stretch of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15 or 18 variable amino acids. In one embodiment, candidate polypeptides further comprise one or more invariable amino acids each, wherein said invariable amino acids can be part of a known consensus binding sequence or provide for at least a part of a scaffold structure. The term “consensus binding sequence” refers to an amino acid sequence comprising variable and invariable amino acids, wherein the invariable amino acids facilitate binding of a polypeptide comprising the consensus binding sequence to a protein of the invention. The identity and position of the invariable amino acids can be known prior to carrying out the method or be determined while carrying out said method by identifying bound polypeptides. For example, the affinity of a weakly-binding polypeptide can be improved by constructing a second generation library. If a consensus binding sequence is observed but the polypeptides bind with low affinity, a new, second generation library can be constructed in which the consensus residues are fixed, i.e. invariable, and the residues flanking them are variable, e.g. randomised.

The term “scaffold structure” refers to a structure within, adjacent to or carrying a polypeptide of the invention which imposes a constraint on the structure of the polypeptide. It can also refer to a polypeptide of the invention itself if it is modified so that its structure is constrained to one or more particular conformations. One example of a scaffold structure is a cyclic polypeptide, e.g. with a disulfide-closed loop formed by polypeptide flanking cysteines (CX_yC, wherein C is cysteine, X is a variable amino acid and y the number of variable amino acids). The smaller the loop the larger is the extent of the constraint. The constraint can also be increased by incorporating the amino acids P, V and/or I in the loop. Another example is a

protein onto which variable positions are grafted, e.g. by randomising surface residues, preferably a protein which has other beneficial features such as high stability, facilitated recognition or a useful metabolic function. Further examples of scaffold structures are minibodies (truncated antibody V_H domains), single-chain-antibody-like polypeptides, bacterial receptors, zinc-finger-scaffolds, protease inhibitors and coiled-coil stem loop miniproteins (Nygren and Uhlen, Current Opinion in Structural Biology 1997, 7:463-469). Scaffold structures can improve the stability, i.e. increase the half-life of the polypeptides of the invention, facilitate their transport across cellular membranes or increase their binding affinity. The latter can be due to a smaller loss of entropy upon binding or by exposing residues that may otherwise be in an unfavourable conformation, e.g. hydrophobic residues may be buried. If comprising a scaffold structure, the polypeptides of the invention can have a size which may exceed above-mentioned limitations on the polypeptide length, depending on the type of scaffold structure. For example, when a protein is utilised as a scaffold structure, the polypeptide including the scaffold structure can have a length of many hundreds amino acids, e.g. up to 100, up to 200, up to, 300, up to 400, up to 500, up to 750, up to 1000 or more amino acids.

In one embodiment, said candidate polypeptides are presented by phage display, i.e. are coupled to a bacteriophage coat protein by ligating the polynucleotide encoding a candidate polypeptide to the gene encoding for said coat protein. The term "phage display" refers to a selection technique in which a library of candidate polypeptides is expressed on the outside of phage virions, while the genetic material encoding each candidate polypeptide resides on the inside. This creates a physical linkage between each candidate polypeptide sequence and the DNA encoding it, which allows rapid partitioning based on binding affinity to a given target molecule by a selection process called panning. In its simplest form, panning is carried out by incubating a library of phage-displayed candidate polypeptides with a plate or bead coated with the immobilised target protein, washing away the unbound phage, and eluting the specifically bound phage. The eluted phage is then amplified and taken through additional binding/amplification cycles to enrich the pool in favour of binding polypeptides. After several rounds, e.g. 3-4, individual clones are characterised, for example by DNA sequencing and ELISA. Non-limiting examples for phages suitable for phage display are M13, fd filamentous phage, T4, T7, and λ phage. Coat proteins are proteins forming the surface of the phage, which can accommodate and display heterologous protein sequences that are cloned on their N- or C-terminus forming fusion proteins. Different coat proteins can be used for this purpose, and with the present invention, principally all phage coat proteins

known in the art can be used, in particular the minor coat protein (also named as coat protein III/3, g3p, gIIIp, p3, pIII, cpIII, or cp3) and the major coat protein (also named as coat protein VIII/8, g8p, gVIIIp, p8, pVIII, cpVIII, or cp8), but also other coat proteins such as cp6, cp7, and cp9.

5 In another embodiment, said candidate polypeptides are presented by mirror phage display, wherein said protein comprises and preferably consists of D-amino acids and said candidate polypeptides comprise and preferably consist of L-amino acids. The underlying principle is that candidate polypeptides binding to said protein can be synthesised using D-amino acids and that these D-Amino acid polypeptides will bind to said protein in its natural
10 form, i.e. made from L-amino acids.

Furthermore, the invention relates to a method of targeting a cell expressing a protein comprising the amino acid sequence according to SEQ ID NO: 2, 3, 4, 5 or 6 or a variant thereof, wherein a variant comprises an amino acid sequence which is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%
15 identical to the amino acid sequence according to one or more of said SEQ ID NOs, using the polypeptides of the invention or a polypeptide isolated with the above-described method for isolating polypeptides.

The term "targeting" refers to releasing said polypeptides into the environment said cell is comprised in so that said polypeptides can attach to said cell via binding said protein.
20 This environment can be any entity a cell can be comprised in, such as any cell culture container, for example flasks, well-plates etc., any liquids, for example body liquids or culture media, or any tissues, organs or body parts of a human or animal body or the human or animal body itself, wherein said tissue, organ, body or body parts can be dead or alive. Accordingly, the targeting can be carried out *ex vivo* or *in vivo*. Preferred organs or body parts are kidney,
25 colorectum, lung, brain, head, neck, breast, uterus, cervix, endometrium and pancreas. Said cell is a prokaryotic or, preferably, a eukaryotic cell. In a preferred embodiment, it is a tumour cell, even more preferably a hypoxic tumour cell.

The invention also relates to the diagnosis, prognosis and/or treatment of a tumour using a polypeptide of the invention, polypeptides isolated with the above-described method
30 of the invention, a polynucleotide of the invention or a vector comprising the same. In a preferred embodiment, the tumour comprises hypoxic tumour cells. In another preferred embodiment, the tumour comprises cells expressing, preferably overexpressing CaIX or fragments thereof, e.g. according to SEQ ID Nos 3, 4, 5, and/or 6. Preferred tumours are renal

cancer, colorectal cancer, lung cancer, glioblastoma, head and neck cancer, breast cancer, uterine, cervix, endometrium cancer or pancreatic cancer tumours.

BRIEF DESCRIPTION OF THE FIGURES

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The following figures are merely illustrative of the present invention and should not be construed to limit the scope of the invention as indicated by the appended claims in any way.

10 **Figure 1:** Structure of a filamentous bacteriophage displaying a variable peptide sequence on its surface: The variable peptide sequence (random 12-mers) is expressed as a fusion with a coat protein (pIII) of the bacteriophage, resulting in display of the fused protein on the surface of the phage.

15 **Figure 2:** Binding and competition of the ^{125}I -labeled CaIX-P1 peptide on the recombinant extracellular domain of human carbonic anhydrase IX (CaIX) and human epidermal growth factor receptor (EGFR). Non-specific binding was determined in the presence of 10^{-4} M unlabeled CaIX-Pep1. Mean values and standard deviation (n=3).

20 **Figure 3:** *In vitro* characterization. (A) Binding of ^{125}I -labeled CaIX-P1 in the CaIX positive human renal cell carcinoma cell line SKRC 52, the human colorectal carcinoma cell line HCT 116, the CaIX negative human renal cell carcinoma cell line CaKi 2 and on human umbilical vein endothelial cells (HUVEC). (B) Displacement of bound ^{125}I -CaIX-P1 by the unlabeled CaIX-P1 peptide at various concentrations in SKRC 52 cells. (C) Specific binding of ^{125}I -CaIX-P1 in SKRC 52 cells. Non specific binding was determined in the presence of 10^{-5} M unlabeled CaIX-Pep1. Octreotide was used at the same concentration (10^{-5} M) as negative control competitor. (D) *In vitro* cell accumulation of ^{125}I -CaIX-P1 in SKRC 52 cells as a function of time. Incubation was performed for time periods from 10 min to 360 min. Mean values and standard deviation (n=3).

30 **Figure 4:** Binding and internalization of ^{125}I -CaIX-P1 in SKRC 52 cells. Cells were incubated with the radioligand for (A) 10 min and (B) 60 min at 37 °C or at 4 °C. Mean values and standard deviation (n=3).

Figure 5: Quantitative RT-PCR analysis of CaIX mRNA in HCT 116 and HUVEC cells. (A) CaIX mRNA levels in HCT 116 cells as function of the cell density. (B) Binding of ^{125}I -CaIX-P1 in HCT 116 cells as function of the cell density. (C) CaIX mRNA levels in HCT 116 and HUVEC cells at the same cell density. (D) Binding of ^{125}I -CaIX-P1 in HCT 116 and HUVEC cells at the same density. Mean values and standard deviation (n=3).

Figure 6: FACS analysis of FITC-CaIX-P1 and rhodamine labelled anti-Ca9-IgG on HCT 116 cells. I) autofluorescence, II) rhodamine-anti-Ca9-IgG labelled cells, III) FITC-CaIX-P1 labelled cells.

Figure 7: Fluorescence microscopy studies of FITC-labelled CaIX-P1 on HCT 116 cells.

Figure 8: Serum stability analysis of CaIX-Pep1. HPLC analysis of aliquots collected at time points from 0 min to 120 min after incubation of CaIX-P1 in human serum at a concentration of 10^{-4} M.

Figure 9: *In vivo* imaging of ^{125}I -CaIX-P1 in a mouse subcutaneously carrying an SKRC 52 tumour in the right thigh.

Figure 10: Organ distribution of ^{131}I -labeled CaIX-P1 in female Balb/c nu/nu mice carrying SKRC 52 tumours. Black bars show the activity concentration (% ID/g) in tumour and control organs after 15 min circulation of ^{131}I -labeled p160 in the mice (n = 3 animals). White bars show the radioactivity concentration (% ID/g) in tumour and control organs after 1 h circulation of the radioligand in the blood stream Mean values and standard deviation (n = 5 animals).

Figure 11: Alanine scanning of ^{125}I -labeled CaIX-P1 peptide on CaIX positive human renal cell carcinoma cell line SKRC 52. Ratio binding-derivative to binding-CaIX-P1.

Figure 12: Binding of the CaIX-P1 peptide fragments CaIX-P1-1-8, CaIX-P1-3-10 and CaIX-P1-5-12 on CaIX positive human renal cell carcinoma cell line SKRC 52.

Figure 13: *In vitro* cell accumulation of ^{125}I -CaIX-P1-4-10 in SKRC 52 and BxPC-3 cells as a function of time. Incubation was performed for time periods from 10 min to 120 min. Mean values and standard deviation (n=3).

5 **Figure 14:** A: Displacement of bound ^{125}I -CaIX-P1-4-10 by the unlabeled CaIX-P1 and CaIX-P1-4-10 peptide at various concentrations in SKRC 52 cells. B: Specific binding of ^{125}I -CaIX-P1-4-10 in SKRC 52 cells. Non specific binding was determined in the presence of 10^{-5} M unlabeled CaIX-P1-4-10 and CaIX-P1. Octreotide was used at the same concentration (10^{-5} M) as a negative control competitor. Mean values and standard deviation (n=3).

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Figure 15: Binding and internalization of ^{125}I -CaIX-P1-4-10 in SKRC 52 cells. Cells were incubated with the radioligand for 10 min, 30 min, 60 min, 120 min and 240 min at 37 °C or at 4 °C. Mean values and standard deviation (n=3).

15 **Figure 16:** Alanine scanning of ^{125}I -labeled CaIX-P1-4-10 peptide in SKRC 52 cells. Ratio binding-derivative to binding-CaIX-P1-4-10. Mean values and standard deviation (n=3).

Figure 17: Deletion scan of ^{125}I -labeled CaIX-P1-4-10 peptide in SKRC 52 cells. Ratio binding-derivative to binding-CaIX-P1-4-10. Mean values and standard deviation (n=3).

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Figure 18: Stability of CaIX-P1-4-10 in cell media from SKRC 52 cells.

Figure 19: Stability of CaIX-P1-4-10 in cell media from BxPC-3 cells.

25 **Figure 20:** Stability of C-terminale fragments of CaIX-P1-3-10 labeled with ^{125}I .

Figure 21: HPLC Chromatogram of C-terminale fragments of CaIX-P1-3-10 and HPLC Chromatogram of supernatant of CaIX-P1-3-10 in cell media of SKRC 52 cells are superimposed.

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Figure 22: Methyl scan of ^{125}I -CaIX-P1-4-10 in SKRC 52 cells as a function of time. Incubation was performed for time periods from 10 min to 120 min. Mean values and standard deviation (n=3).

Figure 23: Organ distribution of ^{131}I -CaIX-P1-4-10 in Balb/c nu/nu mice carrying subcutaneously CAIX positive SKRC52 tumors.

Figure 24: *In vitro* cell accumulation of ^{125}I -labeled derivatives of CaIX-P1-4-10 in SKRC 52 cells as a function of time. Modified derivatives of CaIX-P1-4-10 were synthesized in order to improve the peptide stability and tested for binding on CaIX expressing SKRC 52 cells. Incubation was performed for time periods from 10 min to 120 min. Mean values and standard deviation (n=3). Modifications: n-HVPLSPy: D-asparagine N-terminal, a-HVPLSPy: D-alanine N-terminal, AcN-HVPLSPy: acetylated asparagine N-terminal, betaA-HVPLSPy: beta alanine N-terminal, MeG-HVPLSPy: methylglycine N-terminal.

Figure 25: *In vitro* cell accumulation of ^{125}I -labeled derivatives of CaIX-P1-4-10 in SKRC 52 cells as a function of time. Modified derivatives of CaIX-P1-4-10 were synthesized in order to improve the peptide stability and tested for binding on CaIX expressing SKRC 52 cells. Incubation was performed for time periods from 10 min to 120 min. Mean values and standard deviation (n=3). Modifications: 4-10-FN1: AP in the sequence of NHVPLAP substituted by pyridone, 4-10-FN2: Alanine in the sequence of NHVPLAP substituted by taurin.

Figure 26: *In vivo* imaging of ^{125}I -CaIX-P1-4-10 in a mouse subcutaneously carrying an SKRC 52 tumour in the right thigh.

Figure 27: Intavis CelluSpotsTM Peptide Arrays. Arrays of peptide-cellulose conjugates spotted on glass slides were used for investigation of peptide specificity and identification of derivatives with improved affinity. The peptide CaIX-P1 and modified derivatives were spotted on a glass slide. The slides were incubated with the extracellular domain of human carbonic anhydrase IX and with human carbonic anhydrase II. Detection using an HRP-labeled antibody revealed the target bound spots.

Figure 28: Binding of ^{125}I -CaIX-P1 on immobilized Ca II und Ca IX protein.

EXAMPLES

The following examples are for illustrative purposes only and do not limit the invention described above in any way.

Example 1: Recombinant isolation of the extracellular domain of carbonic anhydrase IX

For recombinant isolation of the extracellular domain of carbonic anhydrase IX (CaIX) the Flp-In system (Invitrogen life technologies) was used. The gene encoding for human carbonic anhydrase IX inserted into a pCMV6-XL5 vector was obtained from Origene, Rockville. The primers for PCR amplification of the sequence encoding for the extracellular domain of CaIX were forward: 5'-AAC TTA AGC TTG GGG CCG CCA CCA TGG CTC CCC TGT GCC CCA-3' and reverse: 5'-GGC TCC GGA TCC ATG TCC CTG CCC TCG ATG TCA CCA GCA GCC AGG CAG-3'. After PCR amplification, the sequence encoding for the extracellular domain of CaIX was inserted into the HindIII and BspEI sites of pSEC-EGP-2-Fc γ vector (Affimed, Heidelberg). The fragment CaIX-Fc was cut by HindIII and XhoI and inserted into pcDNAEpcam vector (Affimed, Heidelberg). This vector was cotransfected with the Flp recombinase expression vector pOG44 into the Flp-InTM-293 human embryonic kidney host cell line, as described in the Flp-In protocol. Thereafter, a selection for hygromycin resistant cells was performed. The expressed protein was isolated and purified from the incubation medium through a HiTrapTMMabSelect SuReTM column. Qualitative control was performed by ELISA and western blot analysis (data not shown).

Example 2: Selection of peptides binding carbonic anhydrase IX using phage display

A linear 12-amino acid peptide library (Ph.D.12; New England Biolabs) was used for biopanning. Panning was performed on immobilized recombinant extracellular domain of human carbonic anhydrase IX. Immobilized recombinant extracellular domain of the epidermal growth factor receptor (EGFR) was used for negative selection. Each selection round was conducted as follows: 10¹¹ plaque-forming units were added on immobilized negative target (EGFR) in 96well plates. After 1h incubation at room temperature medium was transferred in 96wells containing the immobilized positive target (CaIX). Incubation was carried out for 1h at room temperature. Subsequently, medium was removed and the target was washed 10 times with 100 μ l TBST. Elution of the bound phages was performed through incubation for 10 min with 10 μ l 0.2M glycine/HCl buffer pH 2.2, containing 1 mg/mL BSA at room temperature. After neutralization with 15 μ l Tris/HCl buffer pH 9.1, centrifugation was performed for 5 min at 1000 rpm. Supernatant was collected and 10 μ l were used for phage titration on IPTG/X-Gal (Fermentas) lysogeny broth agar plates. The remaining

supernatant was used for amplification in 20 mL of ER2537 bacteria according to the manufacturer's protocol. After 7 selection rounds, clones were picked and phage single-stranded isolation was performed (QIAprep Spin M13 Kit; Qiagen). DNA sequencing was carried out and the displayed peptide was identified through analysis with the HUSAR map (HUSAR Biocomputing Service at the German Cancer Research Center). The selection yielded one peptide with the peptide sequence YNTNHVPLSPKY.

Example 3: Peptide synthesis and radioactive labeling

The peptide CaIX-P1 (YNTNHVPLSPKY) was synthesised by solid phase peptide synthesis using Fmoc coupling protocols. CaIX-P1 was synthesised on an ABI 433 A peptide synthesis reactor (Applied Biosystems). The peptide was purified by high performance liquid chromatography (HPLC) on a Chromolith Semi Prep Column RPe18, 10 × 100 mm (Merck), with a linear gradient of water and acetonitrile containing 0.1% trifluoroacetic acid and subsequent lyophilization. The mass of the product was determined by mass spectrometry analysis on a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-3; Kratos instruments). Labeling with ^{125}I and ^{131}I was performed using the chloramine-T method (Crim JW et al., *Peptides*. 2002;23:2045-51). The iodinated product was purified and analysed on a Chromolith Performance RP-18e 100 × 4.6 mm column (Merck) using a linear gradient of water and acetonitrile containing 0.1% trifluoroacetic acid.

Example 4: Binding experiments on immobilized protein

Binding of ^{125}I -labeled CaIX-P1 was performed on immobilized recombinant extracellular domain of human carbonic anhydrase IX (CaIX) and of epidermal growth factor receptor (EGFR) as negative control. For this purpose the target proteins CaIX and EGFR were incubated at a concentration of 50 nM in 24-well plates for 24 h. The 24-well plates were washed three times with 500 μl PBS pH 7.4. Incubation with ^{125}I -CaIX-P1 was performed in 500 μl PBS pH 7.4 for 30 min. After incubation the plates were washed three times with 500 μl ice cold PBS pH 7.4. The target proteins were degraded with 500 μl NaOH 0.3 M and the radioactivity was counted with a γ -counter. Bound radioactivity was calculated as percentage applied dose. In order to evaluate the specificity of the radioligand binding, competition experiments with the unlabeled CaIX-P1 peptide at a concentration of 10^{-4} M were carried out.

Binding of the radioligand was about 8.5% on the extracellular domain of CaIX. Co-incubation of the radioligand with the unlabeled CaIX-P1 peptide at a concentration of 10^{-4} M led to a binding inhibition of about 93% ($p < 0.01$). Experiments on the negative control EGFR protein revealed a reduced binding to the background level (Fig. 2).

The binding experiments on immobilized protein revealed a higher accumulation on carbonic anhydrase IX, compared to the epidermal growth factor receptor, which was used as negative control protein. This binding was found to be inhibited by the unlabeled CaIX-P1 peptide, a result that shows a specific affinity for the target.

Example 5: *In vitro* binding, competition experiments and kinetic studies

All cell lines were cultivated at 37°C in a 5% CO₂ incubator. The human renal cell carcinoma cell lines SKRC 52 and CaKi 2 as well as the human colorectal carcinoma cell line HCT 116 were cultured in RPMI 1640 with GlutaMAX (Invitrogen) containing 10% (v/v) fetal calf serum (Invitrogen). Primary isolated human umbilical vein endothelial cells (HUVEC: Promocell, Heidelberg, Germany) were cultured in serum reduced (5% fetal calf serum [FCS]) modified Promocell medium (MPM), supplemented with 2 ng/mL VEGF and 4 ng/mL basic fibroblast growth factor (bFGF).

For binding experiments, 3×10^5 SKRC 52 cells were seeded into 6-well plates and cultivated in 3 mL of incubation medium at 37 °C for 24 h. After cell blocking with RPMI 1640 (without FCS) containing 1% BSA, the medium was replaced with 1 mL of fresh medium (without FCS) containing $0.5\text{--}1.5 \times 10^6$ cpm of ¹²⁵I-labeled peptide and incubation was performed for time periods varying from 10 min to 6 h at 37 °C. Cells were incubated with the radioligand in serum free medium in order to avoid peptide degradation. To determine specific versus non-specific binding, the cells were incubated with the unlabeled CaIX-P1 peptide at concentrations varying from 10^{-4} to 10^{-10} mol/L. Octreotide was used as negative control competitor. After incubation the medium was removed and the cells were washed three times with 1 mL ice cold PBS, in order to remove the unbound radiolabeled peptide. Subsequently, the cells were lysed with 0.5 mL NaOH 0.3 mol/L and the radioactivity was measured with a γ-counter. Bound radioactivity was calculated as percentage applied dose per 10^6 cells. Binding experiments were also performed on the cell lines HCT 116 at various cell densities and on human umbilical vein endothelial cells (HUVEC). CaIX negative CaKi-2 cells were used as negative control cell line. Data were analyzed employing the unpaired Student t-test and significance was assumed at $P < 0.05$.

The *in vitro* binding experiments demonstrated the highest uptake for the CaIX positive renal cell carcinoma cell line SKRC 52. In particular, the binding capacity on SKRC 52 cells was about 2.5% applied dose per 10^6 cells after 60 min incubation with the radioligand. Binding of ^{125}I -CaIX-P1 on the colorectal carcinoma cell line HCT 116 was 1.0 to 1.5%. Performing the binding experiments on the CaIX negative human renal cell carcinoma cell line CaKi 2 and on human umbilical vein endothelial cells (HUVEC), the binding capacity was found to be reduced to the background level. In particular about 0.4% of the applied dose per 10^6 cells was measured on CaKi 2 and HUVEC cells (Fig. 3A). The difference in the binding capacity between the positive tumour cell lines SKRC 52 and HCT 116 and the negative control cell lines CaKi 2 and HUVEC was found to be highly significant with $p < 0.01$.

Co-incubation of ^{125}I -labeled CaIX-P1 with the unlabeled peptide in SKRC 52 cells resulted in a concentration dependent inhibition of the radioligand binding. At a competitor concentration of 10^{-4} mol/L a maximal inhibition of about 90% was reached ($p < 0.01$). The IC50 value was calculated as 1-3 μM (Fig. 3B). Using octreotide as negative control competitor at the same concentration as the unlabeled CaIX-Pep1, the uptake of ^{125}I -CaIX-P1 on SKRC 52 cells could not be competitively abolished (Fig. 3C).

Kinetic studies of ^{125}I -CaIX-P1 in SKRC 52 cells, with incubation periods varying from 10 min to 6 h, revealed a time dependent decrease of the radioligand uptake. Particularly, maximal uptake of about 3.5% was reached after an incubation period of 10 min. Thereafter a time-dependent decrease was noticed with the bound activity reaching a value of about 1.5% after 6 h of incubation (Fig. 3D).

The *in vitro* studies on different cell lines revealed results that strengthen the hypothesis of a specific binding on CaIX. The radioligand showed a higher accumulation for the SKRC 52 cell line, which is described in the literature to be positive for CaIX, while the binding capacity of the peptide ligand was reduced to the background level for the CaIX negative cell line CaKi 2 (Hulikova A et al., *FEBS Lett.* 2009;583:3563-8). The specificity of the accumulation of CaIX-P1 was evaluated by competition experiments. Those experiments revealed that the uptake of the radiolabeled peptide in CaIX positive SKRC 52 cells was reduced with increasing concentration of the unlabeled CaIX-P1 peptide, whereas studies using octreotide as unspecific competitor at the same concentration revealed no effect.

Example 6: Correlation between peptide binding and CaIX antibody uptake

For fluorescence activated cell sorting, 1×10^6 HCT 116 cells were seeded into 6-well plates and cultivated in 3 mL of incubation medium (RPMI + 10% FCS) at 37°C for 24 h. The medium was replaced by 1 mL of fresh medium (without fetal calf serum) containing 15 µL mouse anti-hCa9-IgG for 24 h. Thereafter the cells were washed thrice with ice cold PBS and incubated in 1 mL of fresh medium with a rhodamine-labelled anti-Mouse-IgG-Ab for 2h. Cells were also incubated with FITC-CaIX-P1 at a concentration of 5×10^{-6} mol/L for 24 h. After incubation the medium was removed and the cells were washed with 3 mL of incubation medium. The cells were transferred in 1.5 mL Eppendorf-tubes and centrifugated at 1,000 rpm for 10 min. The pellet was resuspended in cell wash medium, and fluorescence activated cell sorting was performed. To discriminate between peptide- or antibody-bound labelled cells and autofluorescence of the unlabelled HCT 116 cells, we measured the autofluorescence of untreated HCT 116 cells. Fluorescence up to the measured intensity was considered autofluorescence and determined through a cut-off line. Cells in which the fluorescence was higher than the cut-off value were considered labelled with FITC-CaIX-P1 or anti-hCa9-IgG. FACS analysis was performed in a Galaxy Pro flow cytometer (Partec) equipped with a mercury vapour lamp (100 W) and filter combinations for FITC and rhodamine. Histogramm and dot blot analysis was done with the FlowMax analysis software (Partec).

The FACS analysis revealed similar results for FITC-CaIX-P1 and anti-hCa9-IgG labelling of the HCT 116 cells at the investigated cell density. In particular, about 36% of the cells were found to be labelled with anti-hCa9-IgG, while about 28% of the cells were found to be labelled with FITC-CaIX-Pep1. The correlation between binding of the CaIX-P1 peptide and the anti-human Ca9-Ab indicates a specificity of the peptide for human carbonic anhydrase IX.

Example 7: Cellular localization of the peptide

For fluorescence studies, 50,000 HCT 116 cells in (RPMI + 10% FCS) were seeded onto coverslips. After 24 h of cultivation, the medium was replaced by fresh medium (without FCS) and FITC-CaIX-P1 was added to the cells at a concentration of 10^{-5} mol/L. The FITC-labelled peptide was incubated with the cells for 60 minutes at 37°C. After incubation, the medium was removed and the cells were washed thrice with 1 mL RPMI medium. Subsequently, the cells were fixed with 2% formaldehyde for 20 min on ice. Finally, the cells were washed thrice with 1 mL PBS and the coverslips were put on slides using fluorescent mounting medium (DAKO, Carpinteria, CA). Samples without FITC-CaIX-P1 were analyzed

to determine autofluorescence. After treatment of the cells, fluorescence microscopy was performed with a Nikon fluorescence microscope (Melville, NY, USA).

Fluorescence microscopy studies revealed an intensive fluorescence signal mainly at the periphery of the cells. To exclude autofluorescence of the HCT 116 cells, investigation of untreated cells was performed, revealing no fluorescence signal (data not shown).

Example 9: Internalization studies

To distinguish between surface bound and internalized peptide, *in vitro* internalization was investigated in SKRC 52 cells. The human renal cell carcinoma cell line SKRC 52 was cultured in RPMI 1640 with GlutaMAX (Invitrogen) containing 10% (v/v) fetal calf serum (Invitrogen) at 37°C in a 5% CO₂ incubator.

Subconfluent cell cultures of SKRC 52 cells were incubated with ¹²⁵I-CaIX-P1 for 10 min and 60 min at 37 °C and 4 °C. Cellular uptake was stopped by removing medium from the cells and washing three times with 1 mL PBS. Subsequently, cells were incubated with 1 mL of glycine-HCl, 50 mmol/L in PBS (pH 2.8) for 10 min at room temperature in order to remove the surface bound activity. The cells were then washed with 3 mL of ice-cold PBS and lysed with 0.5 mL of NaOH. The surface and the internalized radioactivity were measured with a γ-counter and calculated as % of the total uptake at 37 °C.

After 10 min incubation with ¹²⁵I-CaIX-P1 at 37 °C, the internalized radioactivity was measured as 45% of the total bound activity (Fig. 4A), while after 60 min incubation 65% of the total uptake was found to be internalized into the SKRC 52 cells (Fig. 4B). Internalization experiments were also performed at 4 °C demonstrating a reduction of both total and internalized radioactivity.

The internalization of CaIX-P1 by cells highlights the usability of this peptide for targeting tumours. By internalization the peptide accumulates in tumour cells, which multiplies its potential for applications such as imaging or radiotherapy.

Example 10: Real time quantitative PCR and ¹²⁵I-CaIX-P1 binding on HCT 116 and HUVEC cells

All cell lines were cultivated at 37°C in a 5% CO₂ incubator. The human colorectal carcinoma cell line HCT 116 was cultured in RPMI 1640 with GlutaMAX (Invitrogen) containing 10% (v/v) fetal calf serum (Invitrogen). Primary isolated human umbilical vein endothelial cells (HUVEC: Promocell, Heidelberg, Germany) were cultured in serum reduced

(5% fetal calf serum [FCS]) modified Promocell medium (MPM), supplemented with 2 ng/mL VEGF and 4 ng/mL basic fibroblast growth factor (bFGF).

Total cellular RNA was isolated from confluent HCT 116 and HUVEC cells in 10 cm cell culture dishes using the Trizol method (TRIzol Reagent, Invitrogen). Cellular RNA was also isolated from HCT 116 cells in 6-wells at various cell densities. RNA extraction was carried out with a standard phenol-chloroform extraction. RNA concentration was measured with a NanoDrop spectrophotometer (ND-1000 PeqLab Biotechnologie GmbH, Germany). 500 ng was transcribed into DNA using M-MLV reverse transcriptase, 50 pmol random hexamer and 100 pmol of oligo(dT) primers (Promega, Madison, WI, USA). The LightCycler FastStart DNA Master Hybridization Probes kit was used for quantification of relative mRNA transcript levels on a Light Cycler (Roche Applied Sciences), applying the TaqMan methodology. Normalization was performed using β 2-microglobulin as house keeping gene. Primers were obtained from Applied Biosystems (Foster City, CA, USA). Data were analyzed employing the unpaired Student t-test and significance was assumed at $P < 0.05$.

Quantitative real time PCR analysis showed an upregulation of CaIX mRNA in the human colorectal carcinoma cell line HCT 116 with increasing cell density (Fig. 5A). This result correlated with the results of the uptake experiments of the ^{125}I -labeled CaIX-P1 peptide in HCT 116 cells. In particular, radioligand binding was found to increase with increasing cell number in the 6-well plate (Fig. 5B).

RT-PCR analysis demonstrated higher CaIX mRNA levels for HCT 116 cells compared to HUVEC cells at same density (Fig. 5C), which also correlated to the binding of the radiolabeled CaIX-P1 peptide in the two cell lines ($p < 0.05$) (Fig. 5D).

RT-PCR showed a correlation between binding of radiolabeled peptide and CaIX mRNA expression for the colorectal carcinoma cell line HCT 116 and for human umbilical vein endothelial cells. Both mRNA expression of carbonic anhydrase IX and binding capacity of the CaIX-P1 peptide were higher for HCT 116 compared to the HUVEC cells. It also revealed a cell density dependent expression of CaIX, which also correlated to the binding of ^{125}I -labeled CaIX-Pep1.

Example 11: Stability in human serum

The *in vitro* stability of CaIX-P1 was investigated in human serum. The peptide was incubated at 37 °C in human serum at a concentration of 10^{-4} mol/L. At time points varying from 5 min to 2 h aliquots were taken, mixed with equal volume acetonitrile, in order to precipitate serum proteins and centrifuged for 5 min at 13,000 rpm. The supernatant was

analyzed with HPLC. Samples of CaIX-P1 and its fragments in human serum were isolated and analyzed by MALDI-TOF mass spectrometry.

The experiments revealed a degradation of the peptide through serum proteases over time (Fig. 8). Analysis of the samples revealed a half-time of about 25 min. The first degradation product of CaIX-P1 identified by HPLC was isolated and mass spectrometry was performed in order to identify the site of cleavage. The main product of serum degradation appeared after 10 min incubation of the peptide in serum and had a mass of 1270 g/mol. This mass corresponds to an 11 amino acid sequence, lacking a tyrosine. Further investigation revealed that the N-terminal tyrosine was the first amino acid to be cleaved by serum proteases.

Stability experiments in human serum demonstrated moderate degradation of CaIX-P1 through serum proteases. In particular, the mass spectrometry indicated that the N-terminal tyrosine molecule was degraded.

Example 12: Visualising tumour targeting of CaIX-P1 in vivo

A cell suspension of 4×10^6 SKRC 52 cells was injected subcutaneously into the right hind leg of 9-week-old female Balb/c nu/nu mice. Once tumours reached approximately a size of approximately 1 cm^3 the animals were anesthetized and ^{125}I -labelled CaIX-P1 (ca. 7 MBq in 100 μl saline buffer) was injected into the tail vein. At 10 min, 30 min, 1h, 2h, 4h and 24h after radioligand injection, the animals were placed under the collimator of a gamma camera and a whole-body planar image acquisition was performed.

The whole-body planar imaging allowed a visualization of the tumour up to 4 h after injection of the radioligand. Moderate background activity was noticed that might be explained by the deiodination or degradation of the radioligand.

Example 13: Organ distribution studies of CaIX-P1

Organ distribution studies were performed in 9-week-old female Balb/c nu/nu mice, carrying subcutaneously transplanted SKRC 52 tumours. Animals were obtained from Charles River WIGA and housed in VentiRacks (BioZone Global). A cell suspension of 4×10^6 cells in OPTI-MEM (Gibco, Invitrogen Life Technologies) was injected subcutaneously into the mouse trunk and the tumours were grown to a size of 1.0 cm^3 . ^{131}I -labeled CaIX-P1 was injected into the tail vein of the animals (approximately 1 MBq) and at 15 min and 60 min after injection the animals were sacrificed. Tumour, blood and selected tissues (heart, spleen, liver, kidney, muscle, intestinum and brain) were removed, drained of blood, weighed

and the radioactivity was measured in a γ -counter (LB 951G; Berthold Technologies). The organ uptake was calculated as percentage injected dose per gram tissue (% ID/g).

The organ distribution revealed a tumour uptake of 2.9% ID/g tissue at 15 min after intravenous injection of the radioligand. The tumour value remained stable after 60 min circulation in the blood stream. Uptake in tumour was higher than in heart, spleen, liver, muscle, intestinum and brain and almost the same compared to lung (Fig. 10). Only the blood value (5.1% at 15 min and 3.5% at 60 min) and the kidney value (6.2% at 15 min and 3.3% at 60 min) were higher. A trend to a decrease of the uptake with time progression was noticed for the healthy organs, but not for the tumour. The tumour-to-organ ratios showed an increase with time for all organs (Table 1).

TABLE 1: Tumour to organ ratios calculated from the organ distribution of ^{131}I -CaIX-P1 in female Balb/c nu/nu mice carrying SKRC 52 tumours after 15 min (n = 3 animals) and after 1 h (n = 5 animals) circulation of the radioligand in the blood stream.

Tumour to organ Ratio	15 min	60 min
Blood	0.557 ± 0.064	0.826 ± 0.229
Heart	1.800 ± 0.079	3.571 ± 2.435
Lung	0.747 ± 0.095	1.756 ± 1.671
Spleen	1.216 ± 0.127	2.110 ± 1.461
Liver	1.324 ± 0.039	2.433 ± 1.305
Kidney	0.463 ± 0.051	1.037 ± 0.645
Muscle	2.624 ± 0.175	5.113 ± 3.3
Intestinum	1.346 ± 0.326	1.511 ± 0.477
Brain	7.177 ± 0.561	23.99 ± 13.174

A prerequisite for the use of a ligand as tracer for imaging purposes is a higher *in vivo* accumulation in tumour tissue, compared to the healthy organs. The results of the organ distribution studies demonstrated that the peptide CaIX-P1 fulfils this criterion. In particular, experiments in nude mice bearing SKRC 52 tumours subcutaneously revealed a higher uptake in tumour than in most of the healthy organs. Only the values in blood, lung and kidney were higher. The enhanced uptake in the kidney can be explained through a rapid renal elimination of the peptide. Such pharmacokinetic properties are favorable for the use of a molecule as an imaging agent since they prevent the long circulation of the drug in blood stream and an

accumulation in healthy tissues. The high blood value might be explained through an interaction of the peptide with serum proteins, such as albumin. A further explanation might be a deiodination of the radioligand. *In vivo* deiodination of directly radiolabeled peptides has been described in the literature (Bakker WH et al., *J Nucl Med.* 1990;31:1501-9). In case of
5 CaIX-P1 the high blood value is additionally explained by the metabolic properties of the peptide. Stability experiments in human serum demonstrated a moderate degradation of CaIX-P1 through serum proteases. Mass spectrometry revealed a degradation of the N-terminal tyrosine molecule. Since direct iodination is performed on the side group of tyrosine, the degradation might lead to free ¹²⁵I-labeled tyrosine residues that circulate in the bloodstream,
10 which may be partly responsible for the observed background signal.

Example 14: Identification of the binding site in the sequence of CaIX-P1

All cell lines were cultivated at 37°C in a 5% CO₂ incubator. The human renal cell carcinoma cell line SKRC 52 was obtained by O. Boerman (Univ. of Nijmegen, The
15 Netherlands). SKRC 52 was cultured in RPMI-1640 with GlutaMAX (Invitrogen) containing 10% (v/v) fetal calf serum (Invitrogen). BxPC-3 was cultured in RPMI-1640 with extra D-Glucose (4.5 g/L) (Invitrogen) containing 10% (v/v) fetal calf serum.

All peptides were obtained by solid phase peptide synthesis on an ABI 433 A peptide synthesis reactor (Applied Biosystems) using Fmoc coupling protocols. Purification was
20 performed by high performance liquid chromatography (HPLC) on a Chromolith Semi Prep Column RPe18, 10 × 100 mm (Merck), with a linear gradient of water and acetonitrile containing 0.1% trifluoroacetic acid and subsequent lyophilization. The mass of the products was determined by mass spectrometry analysis on a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-3; Kratos instruments). Labeling with
25 ¹²⁵I and ¹³¹I was performed using the chloramine-T method [30]. The iodinated products were purified and analyzed on a Chromolith Performance RP-18e 100 × 4.6 mm column (Merck) using a linear gradient of water and acetonitrile containing 0.1% trifluoroacetic acid.

Data were analyzed employing the paired two-tailed Student t-test and significance was assumed at P<0.05.

30 To identify the binding site in the sequence of CaIX-P1 and determine which amino acids are responsible for target affinity, alanine scanning was performed. For alanine scanning, derivatives of CaIX-P1 were synthesized with exchange of each amino acid by alanine. All derivatives were labeled with ¹²⁵I and tested for binding in comparison to radiolabelled native CaIX-P1 on carbonic anhydrase IX positive renal cell carcinoma SKRC

52 cells (Figure 11). The results of alanine scanning indicate that the amino acids ^3T , ^4N , ^8L , ^9S and ^{12}Tyr might be important for CaIX-P1 binding on SKRC 52 cells. To prove this hypothesis, 8-amino acid fragments of the peptide were synthesized, labeled with ^{125}I and investigated for binding on target cells. 8-amino acid fragments were chosen because the peptide CaIX-P1-3-10, representing the middle part of CaIX-P1 is the smallest derivative containing all amino acids that were considered by alanine scanning to be important for ligand binding. Comparison after binding saturation of the ^{125}I -labeled-fragments CaIX-P1-1-8 (YNTNHVPL), CaIX-P1-3-10 (TNHVPLSPy) and CaIX-P1-5-12 (HVPLSPKY) with the native CaIX-P1 (YNTNHVPLSPKY) peptide revealed that the fragment CaIX-P1-3-10 had an up to five-fold higher binding capacity compared to the leader peptide on SKRC 52 cells. The fragments CaIX-P1-1-8 and CaIX-P1-5-12 showed a significantly reduced binding activity (Figure 12).

To further investigate the binding site in the sequence of CaIX-P1, several peptide fragments were synthesized and tested on SKRC52 cells. The ratios binding derivative to binding CaIX-P1 are presented in Table 2.

Table 2: Various fragments of ^{125}I -labeled CaIX-P1 peptide were tested on CaIX positive human renal cell carcinoma cell line SKRC 52. Ratio binding-fragment to binding-CaIX-P1.

Peptide	Sequence	Ratio binding- derivative to binding-CaIX-P1 (10 min)	Ratio binding- derivative to binding-CaIX-P1 (30 min)
CaIX-P1	YNTNHVPLSPKY	1	1
CaIX-P1-3-6	TNHVy	0.285	0.794
CaIX-P1-3-7	TNHVPy	0.600	0.750
CaIX-P1-3-8	TNHVPLy	0.764	1.533
CaIX-P1-3-9	TNHVPLSy	1.568	2.549
CaIX-P1-3-10	TNHVPLSPy	5.400	2.800
CaIX-P1-4-7	NHVPy	0.791	2.610
CaIX-P1-4-8	NHVPLy	0.822	1.569
CaIX-P1-4-9	NHVPLSy	2.622	2.504
CaIX-P1-4-10	NHVPLSPy	5.836	3.019
CaIX-P1-5-9	HVPLSy	0.059	0.209
CaIX-P1-5-10	HVPLSPy	1.539	3.650
CaIX-P1-5-11	HVPLSPKy	0.095	0.170
CaIX-P1-6-10	VPLSPy	0.246	0.694
CaIX-P1-8-10	LSPy	0.044	0.088

These data demonstrated that the peptide CaIX-P1-4-10 had an almost six-fold binding on SKRC52 cells, reaffirming the previous hypothesis that the binding site in the sequence of CaIX-P1 might be between ³Thr and ¹⁰Pro.

5 **Example 15: *In vitro* and *in vivo* evaluation of CaIX-P1-4-10**

For binding experiments 5×10^5 SKRC 52 or BxPC3 cells were seeded into 6-well plates and cultivated in 3 mL of incubation medium at 37 °C for 24 h. After cell blocking with RPMI 1640 (without FCS) containing 1% BSA, the medium was replaced with 1 mL of fresh medium (without FCS) containing $0.8\text{--}1.2 \times 10^6$ cpm of ¹²⁵I-labeled peptide and incubation
10 was performed for time periods varying from 10 min to 2 h at 37 °C. To determine specific versus nonspecific binding, the cells were incubated with unlabeled competitors at concentrations varying from 10^{-4} to 10^{-10} mol/L. Octreotide was used as negative control competitor. After incubation the medium was removed and the cells were washed three times
15 with 1 mL ice cold PBS in order to remove the unbound radiolabeled peptide. Subsequently, the cells were lysed with 0.5 mL NaOH 0.3 mol/L and the radioactivity was measured with a γ -counter. Bound radioactivity was calculated as percentage applied dose per 10^6 cells. CaIX negative BxPC-3 cells were used as negative control. Data were analyzed employing the paired two-tailed Student t-test and significance was assumed at $P < 0.05$.

Since the peptide CaIX-P1-4-10 showed a significantly higher binding than CaIX-P1,
20 *in vitro* kinetic, competition and internalization studies as well as *in vivo* distribution experiments were performed. Binding kinetics of ¹²⁵I-CaIX-P1-4-10 in SKRC 52 cells, with incubation periods varying from 10 min to 2 h, revealed a time dependent decrease of the radioligand binding. Particularly, maximal uptake of 20.7 % was reached after an incubation period of 10 min. Thereafter a time-dependent decrease was noticed with the bound activity
25 reaching a value of about 1.3 % after 2 h incubation. No binding was measured on CaIX negative BxPC-3 cells (Figure 13). To further verify the binding affinity and specificity, competition studies were carried out. Co-incubation of ¹²⁵I-CaIX-P1-4-10 with unlabeled CaIX-P1 or unlabelled CaIX-P1-4-10 on SKRC 52 cells resulted in a concentration dependent inhibition of the radioligand binding with a maximal inhibition of over 95 % at 10^{-4} mol/L
30 competitor concentration (Figure 14 A). At concentration of 10^{-5} mol/L CaIX-P1-4-10 showed a binding inhibition of about 95% and CaIX-P1 of about 80%. The negative control competitor octreotide caused only a slight inhibition of radioligand binding (Figure 14 B), a result that supports the hypothesis of specific binding to the target..

Example 15: Internalization experiments of CaIX-P1-4-10

Subconfluent cell cultures of SKRC 52 cells were incubated with ^{125}I -labeled peptide for 10, 30, 60, 120 and 240 min at 37 °C and 4 °C. Cellular uptake was stopped by removing the medium and washing three times with 1 mL PBS. Subsequently, cells were incubated with 1 mL of glycine-HCl, 50 mmol/L in PBS (pH 2.2) for 10 min at room temperature in order to remove the surface bound activity. The cells were then washed with 3 mL of ice-cold PBS and lysed with 0.5 mL of NaOH 0.3 mol/L. The surface and the internalized radioactivity were measured with a γ -counter and calculated as % applied dose/ 10^6 cells. Data were analyzed employing the paired two-tailed Student t-test and significance was assumed at $P < 0.05$.

To distinguish between surface bound and internalized peptide, *in vitro* internalization was investigated in SKRC 52 cells. After 10 min incubation with ^{125}I -CaIX-P1-4-10 at 37 °C, the internalized radioactivity was measured as about 20% of the total bound activity, while after 60 min incubation 17% of the total uptake was found to be internalized into the SKRC 52 cells. With time progression both membrane bound and internalized radioactivity decreased. Internalization experiments were also performed at 4 °C demonstrating a reduction of both total and internalized radioactivity (Figure 15). These results support the hypothesis of internalization via endocytosis.

Example 16: Identification of the binding site in the sequence of CaIX-P1-4-10

To further characterize the binding region in the sequence of CaIX-P1-4-10, alanine and deletion scanning were performed. For alanine scanning, the amino acids were gradually replaced with alanine. The peptide was labeled with ^{125}I and tested on SKRC 52 cells. Derivative binding was compared to the native peptide. This comparison revealed a significant binding decrease for almost all derivatives at 10 min. After 30 min an increase was noticed for all derivatives, except of ^7Pro (Figure 16). In addition to alanine scanning, deletion scanning was performed. For deletion scanning individual amino acids were deleted, and derivative binding was compared to the binding of native CaIX-P1-4-10 (Figure 17). Deletion scan demonstrated a significant binding decrease of all derivatives after 10 min incubation. Thus, both alanine and deletion scanning showed a significant decrease of the peptide affinity for almost all amino acids. Furthermore, discrepancies were shown for the single amino acids between alanine and deletion scanning. These results indicate that each amino acid probably contributes to the structural conformation, which is necessary for target binding.

Data were analyzed employing the paired two-tailed Student t-test and significance was assumed at $P < 0.05$.

Example 17: Stabilization of ^{125}I -labeled CaIX-P1-4-10 on SKRC 52 cells

5 The stability of CaIX-P1-4-10 was investigated in cell medium *in vitro*. ^{125}I -labelled peptide was incubated at 37 °C on SKRC52 and BxPC3 cells. At time points varying from 10 min to 2 h aliquots were taken and centrifuged for 5 min at 13,000 rpm. The supernatant was analyzed with HPLC. The method was $\text{H}_2\text{O}:\text{CH}_3\text{CN}$, 0-30% in 10 min.

10 The decreasing binding kinetics of the native CaIX-P1 peptide can be explained by an intracellular degradation of the peptide. To investigate this hypothesis medium stability of CaIX-P1-4-10 was investigated after incubation on SKRC 52 (Figure 18) and BxPC-3 (Figure 19) cells. Medium stability studies demonstrated a time dependent degradation of CaIX-P1-4-10 in SKRC52 cells. In particular, only half of the activity was measured after 30 min incubation, while at 120 min incubation radiolabelled CaIX-P1-4-10 was completely
15 degraded. On the contrary, no degradation of the radioligand was shown in CAIX negative BxPC3 cells (Figure 19). To evaluate the degradation site in the sequence of CaIX-P1-3-10 and CaIX-P1-4-10, HPLC chromatograms of the C-terminal fragments of the peptide were analyzed (Figure 20). The HPLC analysis of the C-terminal fragments of CaIX-P1-4-10 was compared to the chromatograms of the cell medium supernatants. This comparison revealed
20 that the peptide was degraded between ^6S and ^7P and the last fragment was Py (Figure 21). The time dependent reduction of cellular binding of ^{125}I -labeled CaIX-P1-4-10 and CaIX-P1 might be explained by processes of intracellular dehalogenation or degradation, leading to radioactive products that are excreted by the cells (Thiry A et al. (2006), Trends Pharmacol Sci 27: 566-573). The HPLC chromatograms strengthened this hypothesis, since they
25 demonstrated *in vitro* peptide degradation with time progression. Furthermore, these experiments led to the identification of the cleavage site, which is of high importance, since it allows targeted modifications that could improve metabolic stability.

To improve metabolic stability of CaIX-P1-4-10, methyl scan was carried out. For methyl scan the amino groups of the amino acids were methylated. All synthesized
30 derivatives were labelled with ^{125}I on the side group of a C-terminal D-tyrosine and kinetic experiments were performed on SKRC 52 cells. The results of these experiments are shown in Figure 22. Methylation of ^6S led to improvement of binding kinetics with increasing peptide binding for incubation periods of up to 60 min. Thus, these results show an increased stability when ^6Ser is methylated. Further chemical methods that can be applied for improvement of

serum stability include peptide cyclization (Li P, Roller PP (2002), Curr Top Med Chem 2: 325-341), targeted exchange of amino acids with unnatural amino acids, such as D-amino acids, which are not recognized by serum proteases (Fischer PM (2003), Curr Protein Pept Sci 4: 339-356), peptide acetylation (John H et al. (2008), Eur J Med Res 13: 73-78), pegylation (Lee SH et al. (2005), Bioconjug Chem 16: 377-382) or grafting the binding motif into a stable scaffold structure (Boy RG et al. (2010), Mol Imaging Biol 12: 377-385)

Example 18: Organ distribution studies of CaIX-P1-4-10

Organ distribution studies were performed in 9-week-old female Balb/c nu/nu mice, carrying subcutaneously transplanted SKRC 52 tumors. Animals were obtained from Charles River WIGA and housed in VentiRacks (BioZone Global). A cell suspension of 6×10^6 cells in OPTI-MEM (Gibco, Invitrogen Life Technologies) was injected subcutaneously into the mouse trunk and the tumors were grown to a size of 1.0 cm³. ¹³¹I-labeled CaIX-P1-4-10 was injected into the tail vein of the animals (approximately 1 MBq) and at 15 min, 60 min and 240 min after injection the animals were sacrificed. Tumor, blood and selected tissues (heart, spleen, liver, kidney, muscle, intestinum and brain) were removed, drained of blood, weighed and the radioactivity was measured in a γ -counter (LB 951G; Berthold Technologies) Also 3 aliquots of the tracer solution used for injection were measured. The organ uptake was calculated as percentage injected dose per gram tissue (% ID/g). Data were analyzed employing the paired two-tailed Student t-test and significance was assumed at P<0.05.

Organ distribution experiments of ¹³¹I-labeled CaIX-P1-4-10 were performed in female Balb/c nu/nu mice, carrying subcutaneously transplanted SKRC 52 tumors. The biodistribution revealed a tumor uptake of 2.5% ID/g tissue at 15 and 60 min after intravenous injection of the radioligand. Only blood and kidney showed a higher uptake than the tumor. At 60 min after intravenous injection uptake in tumor was significantly higher than in most healthy organs (heart, spleen, liver, muscle, brain). Thereafter a significant decrease was noticed (Figure 23). Thus, organ distribution experiments of CaIX-P1-4-10 demonstrate a higher uptake in tumor than in most healthy organs, which is favorable for imaging purposes. The high blood value can be explained by a certain degree of peptide instability, leading to radiolabelled fragments that circulate in the blood stream. The high kidney values can be explained by renal elimination, which is expected for small peptide ligands.

Example 19: Stability in human serum

The stability of CaIX-P1-4-10 was investigated in human serum. ^{125}I -labelled peptide was incubated at 37 °C in human serum. At time points varying from 5 min to 2 h aliquots were taken, mixed with equal volume acetonitrile to precipitate serum proteins and centrifuged for 5 min at 13,000 rpm. The supernatant was analyzed with HPLC.

5

Example 20: Visualising tumour targeting of CaIX-P1-4-10 *in vivo*

A cell suspension of 4×10^6 SKRC 52 cells was injected subcutaneously into the right hind leg of 9-week-old female Balb/c nu/nu mice. Once tumours reached approximately a size of approximately 1 cm^3 the animals were anesthetized and ^{125}I -labelled CaIX-P1-4-10 (ca. 7 MBq in 100 μl saline buffer) was injected into the tail vein. At 10 min, 30 min, 1h and 2h after radioligand injection, the animals were placed under the collimator of a gamma camera and a whole-body planar image acquisition was performed.

The whole-body planar imaging allowed a visualization of the tumour up to 2 h after injection of the radioligand. Moderate background activity was noticed that might be explained by the deiodination or degradation of the radioligand.

15

Example 21: Binding specificity of CaIX-P1

The peptide CaIX-P1 and various derivates of it were spotted on the cellulose membrane. The arrays were incubated with CA IX and CA II and detected by HRP-labeled antibody. Carbonic anhydrase II is expressed in erythrocytes. No binding was detected on CA II (see Fig. 27). Arrays of peptide-cellulose conjugates spotted on glass slides were used for investigation of peptide specificity and identification of derivatives with improved affinity. The peptide CaIX-P1 and various derivates of it were spotted on the cellulose membrane. The arrays were incubated with CA IX and CA II and detected by HRP-labeled antibody. Spots, indicating protein binding were detected only for CaIX. No binding was detected for CA II. CA II is known to be expressed in erythrocytes. The fact that the peptide binds CaIX but not CaII is of high importance, since it might lead to minimizing background when using the peptide for imaging purposes.

20

Binding on Ca IX was significantly higher than binding on Ca II. Binding on Ca II represents unspecific binding reduced to background level (see Fig. 28). [Binding experiments of ^{125}I -CaIX-P1 on immobilized Ca IX and Ca II protein revealed a significantly higher binding for the extracellular domain of human carbonic anhydrase IX. In particular, binding on immobilized Ca II reached the level of unspecific binding and was similar to the level of background binding.

30

CLAIMS

1. A polypeptide comprising an amino acid sequence according to SEQ ID NO: 1 (Y₁N₂T₃N₄H₅V₆P₇L₈S₉P₁₀K₁₁Y₁₂) or a sequence variant thereof, wherein said
5 sequence variant has, with respect to SEQ ID NO: 1, up to 6 amino acid deletions and/or substitutions, preferably conservative substitutions.
2. The polypeptide of claim 1, wherein said variant has an amino acid sequence according to the following formula (I):
10 Y-X₁-X₂-X₃-H-X₄-P-L-X₅-P-X₆-Y (SEQ ID NO : 7),
wherein X₁ is N or Q, X₂ is T or S, X₃ is N or Q, X₄ is V, A, L or I, X₅ is S or T and X₆ is K or R.
3. The polypeptide of claims 1 or 2, wherein said sequence variant has one of the
15 following:
 - (a) up to 6 amino acid deletions,
 - (b) up to 5 amino acid deletions and optionally one amino acid substitutions, or,
 - (c) up to 4 amino acid deletions and optionally up to two amino acid substitutions,
- 20 4. The polypeptide of any one of claims 1 to 3, wherein said deletions are terminal deletions.
5. The polypeptide of any of claims 1 to 4, wherein said sequence variant has up to 3 N-terminal amino acid deletions and up to 3 C-terminal amino acid deletions, up to 4 N-terminal amino acid deletions and up to 2 C-terminal amino acid deletions, or up to 2
25 N-terminal amino acid deletions and up to 4 C-terminal amino acid deletions, and wherein, preferably, the sequence variant has a sequence according to SEQ ID NO: 8 (N₄H₅V₆P₇L₈S₉), SEQ ID NO: 9 (T₃N₄H₅V₆P₇L₈), or SEQ ID NO: 10 (H₅V₆P₇L₈S₉P₁₀), respectively.
30
6. The polypeptide of any of claims 1 to 5, wherein said sequence variant has up to 4 N-terminal amino acid deletions and up to 1 C-terminal amino acid deletion, up to 3 N-terminal amino acid deletions and up to 2 C-terminal amino acid deletions, up to 2 N-terminal amino acid deletions and up to 3 C-terminal amino acid deletions, or up to 1

N-terminal amino acid deletion and up to 4 C-terminal amino acid deletions, and wherein, if applicable, said sequence variant optionally has 1 non-terminal deletion, and wherein, preferably, the sequence variant has a sequence according to SEQ ID NO: 11 (H₅V₆P₇L₈S₉P₁₀K₁₁), SEQ ID NO: 12 (N₄H₅V₆P₇L₈S₉P₁₀), SEQ ID NO: 13 (T₃N₄H₅V₆P₇L₈S₉) or SEQ ID NO: 14 (N₂T₃N₄H₅V₆P₇L₈), respectively.

7. The polypeptide of any of claims 1 to 6, wherein said sequence variant has up to 2 N-terminal amino acid deletions and up to 2 C-terminal amino acid deletions, up to 1 N-terminal amino acid deletion and up to 3 C-terminal amino acid deletions, or up to 3 N-terminal amino acid deletions and up to 1 C-terminal amino acid deletion, and wherein, if applicable, said sequence variant optionally has 1 non-terminal deletion, and wherein, preferably, the sequence variant has a sequence according to SEQ ID NO: 15 (T₃N₄H₅V₆P₇L₈S₉P₁₀), SEQ ID NO: 16 (N₂T₃N₄H₅V₆P₇L₈S₉), or SEQ ID NO: 17 (N₄H₅V₆P₇L₈S₉P₁₀K₁₁), respectively.

8. The polypeptide of any one of claims 1 to 7, wherein the amino acids H₅V₆ or V₆P₇ or preferably H₅V₆P₇ and the amino acid N₄ or L₈, preferably L₈S₉ or more preferably L₈S₉P₁₀ are not deleted.

9. The polypeptide of any one of the preceding claims, wherein not more than two amino acids in total are substituted, conservatively substituted and/or non-terminally deleted.

10. The polypeptide of claim 9, wherein said non-terminal deletion is selected from the group consisting of N₂/K₁₁, T₃/P₁₀, S₉, L₈, P₁₀, N₄, P₇, V₆, and H₅ in order of preference, wherein / indicates equal preference.

11. The polypeptide of claim 9, wherein said substitution and/or said conservative substitution concerns the amino acid selected from the group consisting of Y₁/N₂/T₃/K₁₁/Y₁₂, N₄, H₅, S₉, P₇, V₆, P₁₀, and L₈ in order of preference, wherein / indicates equal preference.

12. The polypeptide of any one of the preceding claims, wherein, if applicable, the amino acid S₉ according to SEQ ID NO: 1 is methylated.

13. The polypeptide of any one of the preceding claims, wherein said substitutions are substitutions with unnatural amino acids and/or up to 4, 5, 6, 7, 8, 9, 10, 11, or 12 amino acids, including substituted/conservatively substituted amino acids, are replaced with their D-amino acid counterpart.

5

14. The polypeptide of any one of the preceding claims, wherein said polypeptide is up to 100, preferably up to 50, more preferably up to 25, more preferably up to 15, and most preferably up to 12 amino acids long, and/or wherein said amino acid sequence is at least 6, 7, 8, 9, 10, 11, or 12 amino acids long.

10

15. The polypeptide of any one of the preceding claims, wherein said polypeptide is labelled; coupled directly or indirectly to one or more tags, chelators, imaging agents and/or therapeutic agents; or is modified by any of the means of the group consisting of substituting one or more atoms with radioactive isotopes, cyclisation, acetylation, pegylation, N-methylation, protecting an N-terminal tyrosine with a t-butyloxycarbonyl group, and providing said polypeptide with a scaffold structure.

15

16. The polypeptide of any one of the preceding claims, wherein said polypeptide is capable of binding to a protein comprising the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 3 SEQ ID NO: 4, SEQ ID NO: 5 and/or SEQ ID NO: 6 or a variant thereof, wherein said variant comprises an amino acid sequence which is at least 80% identical to the amino acid sequence according to one or more of said SEQ ID NOs.

20

- 25 17. A polynucleotide encoding for the polypeptide of any one of claims 1 to 18.

18. A vector comprising the polynucleotide of claim 19.

19. A method for isolating polypeptides which bind to a protein comprising the amino acid sequence according to SEQ ID NO: 2, SEQ ID NO: 3 SEQ ID NO: 4, SEQ ID NO: 5 and/or SEQ ID NO: 6 or a variant thereof, wherein a variant comprises an amino acid sequence which is at least 80% identical to the amino acid sequence according to one or more of said SEQ ID NOs, comprising the steps of:
(i) contacting said protein with a library of candidate polypeptides,

30

(ii) separating unbound polypeptides from said protein, and
(iii) optionally eluting bound polypeptides from said protein and optionally repeating steps (i) to (iii) after enriching the eluted polypeptides,
(iv) identifying the remaining polypeptides bound to or eluted from said protein and optionally repeating steps (i) to (iv).

20. The method of any one of claim 19, wherein said candidate polypeptides each comprise at least one continuous stretch of variable amino acids with a length of 1-60 amino acids.

21. The method of claim any one of claims 19 to 20, wherein said candidate polypeptides further comprise one or more invariable amino acids each.

22. The method of any one of claims 19 to 21, wherein said candidate polypeptides are presented by phage display or mirror phage display, wherein for mirror phage display said protein comprises and preferably consists of D-amino acids.

23. The method of any one of claims 19 to 22, further comprising before step (i) a negative selection, comprising the following steps:

(a) contacting said library of candidate polypeptides with a negative target protein or a domain thereof,

(b) separating unbound polypeptides from said negative target protein or domain thereof,

wherein said negative target protein or domain thereof does not comprise the amino acid sequence according to SEQ ID NO: 2, 3, 4, 5 or 6 or a variant thereof, wherein a variant comprises an amino acid sequence which is at least 80% identical to the amino acid sequence according to one or more of said SEQ ID NOs, wherein only the unbound polypeptides of step (b) are further processed in step (i), and wherein steps (a) and (b) are optionally repeated with the optional repeats of steps (i) to (iii) or (i) to (iv).

24. The method of claim 23, wherein said negative target protein is a receptor protein or comprises the extracellular domain of a receptor protein.

25. The method of any one of claims 23 to 24, wherein said negative target protein is expressed, preferably overexpressed, in tumours.

26. A method of targeting a cell expressing a protein comprising the amino acid sequence according to SEQ ID NO: 2, 3, 4, 5 and/or 6 or a variant thereof, wherein a variant comprises an amino acid sequence which is at least 80% identical to the amino acid sequence according to one or more of said SEQ ID NOs, using the polypeptide of any one of claims 1 to 16 or a polypeptide isolated with the method of any one of claims 19 to 25.

27. The polypeptide of any one of claims 1 to 16, a polypeptide isolated with the method of any one of claims 19 to 25, the polynucleotide of claim 17 or the vector of claim 18 for the use in diagnosis, prognosis and/or treatment of a tumour.

Figure 1

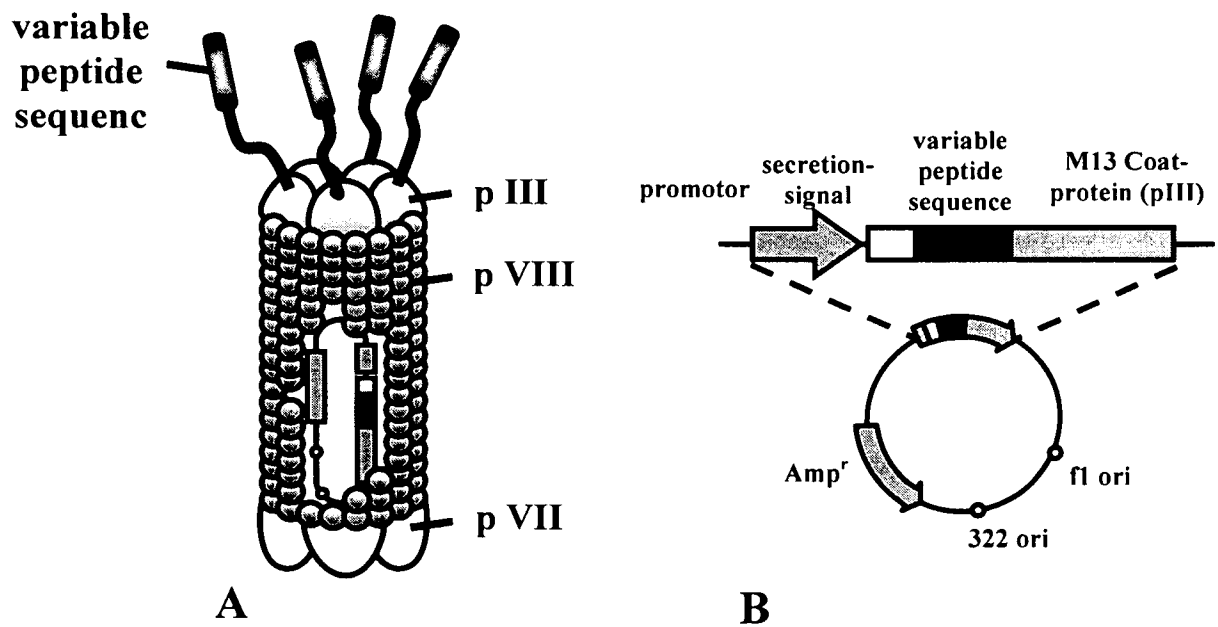


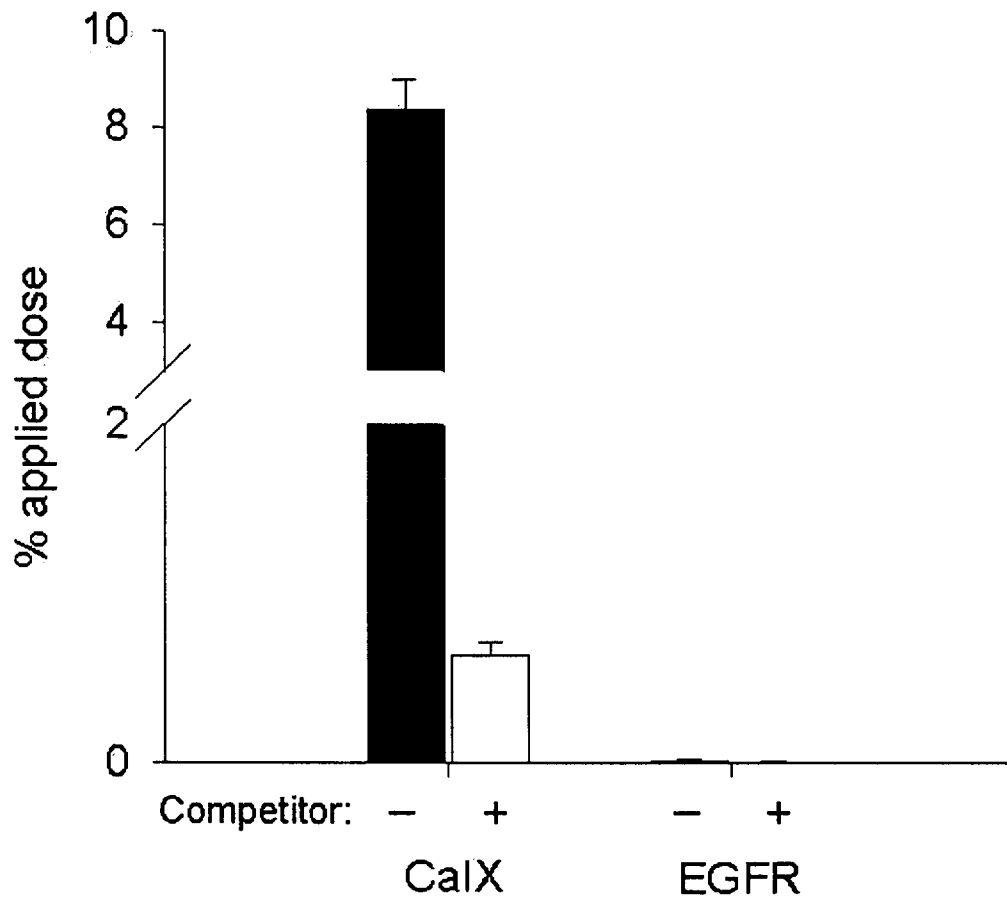
Figure 2

Figure 3

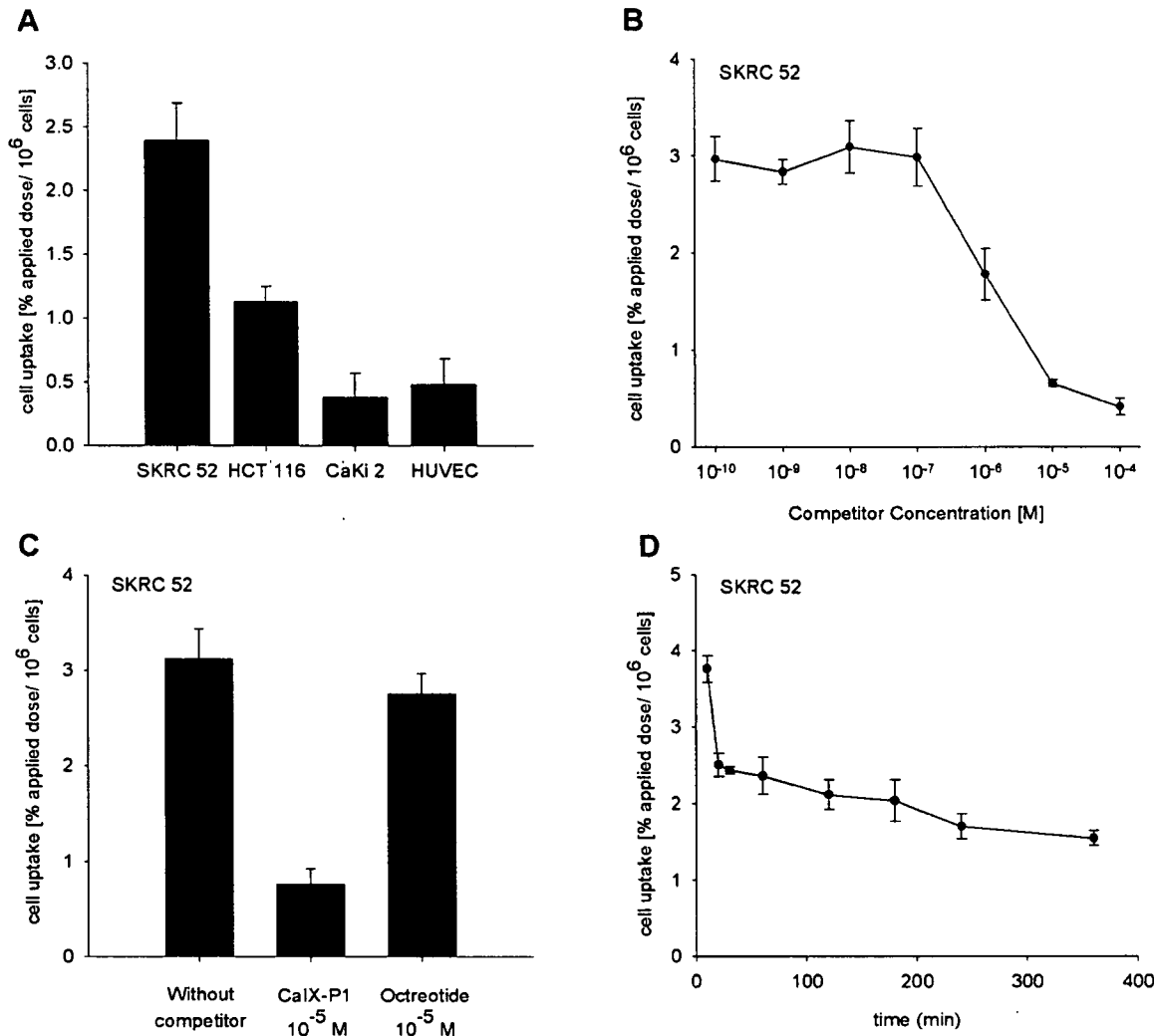


Figure 4

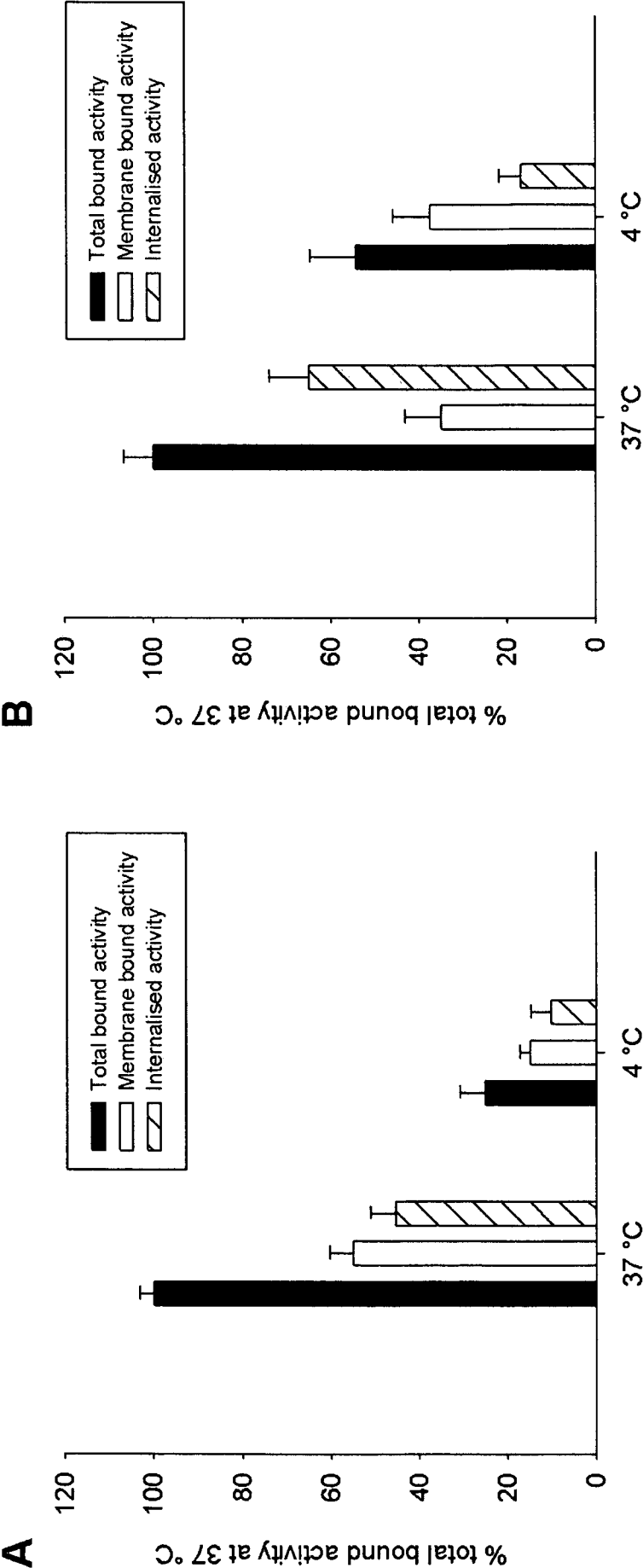


Figure 5

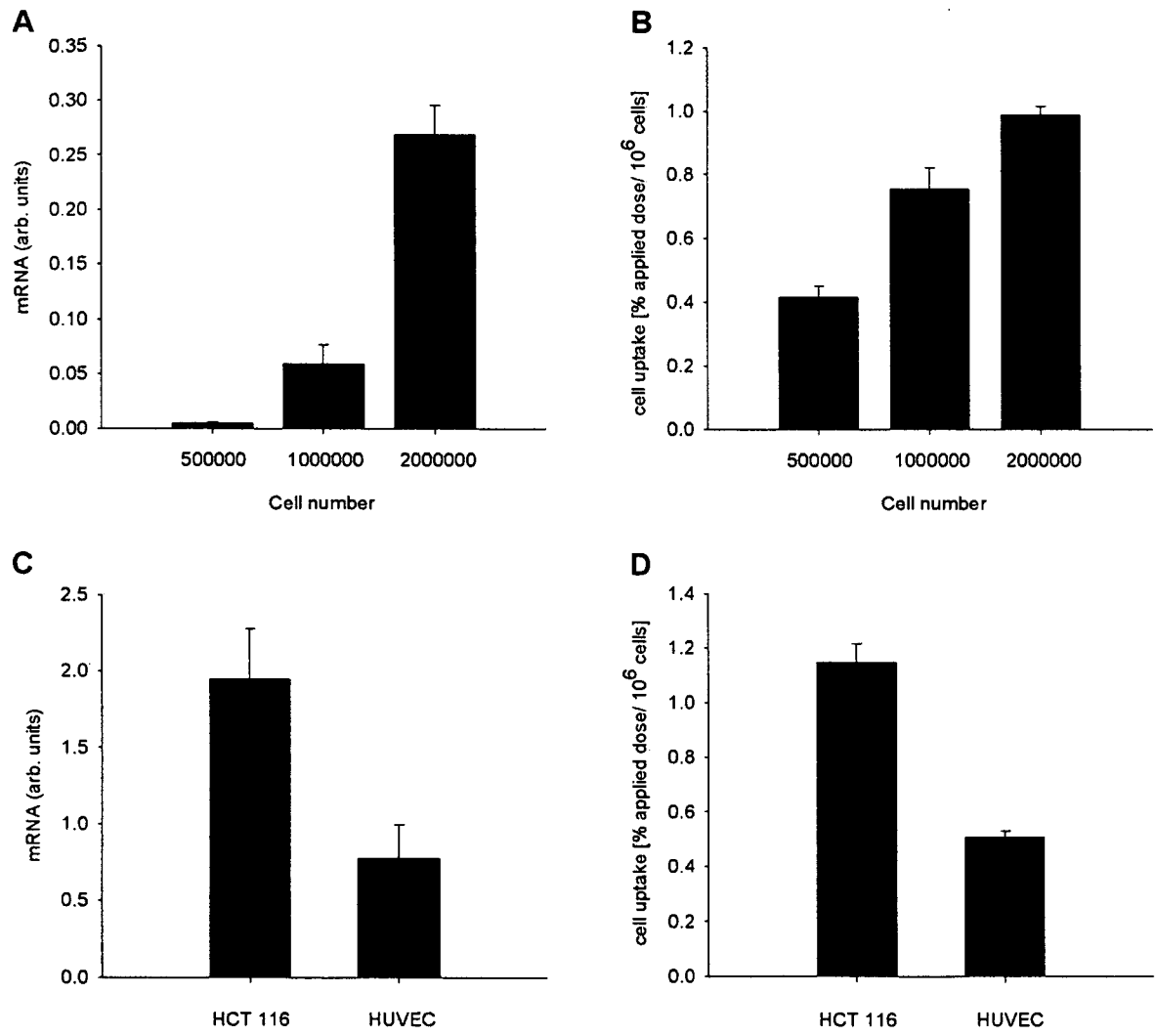


Figure 6

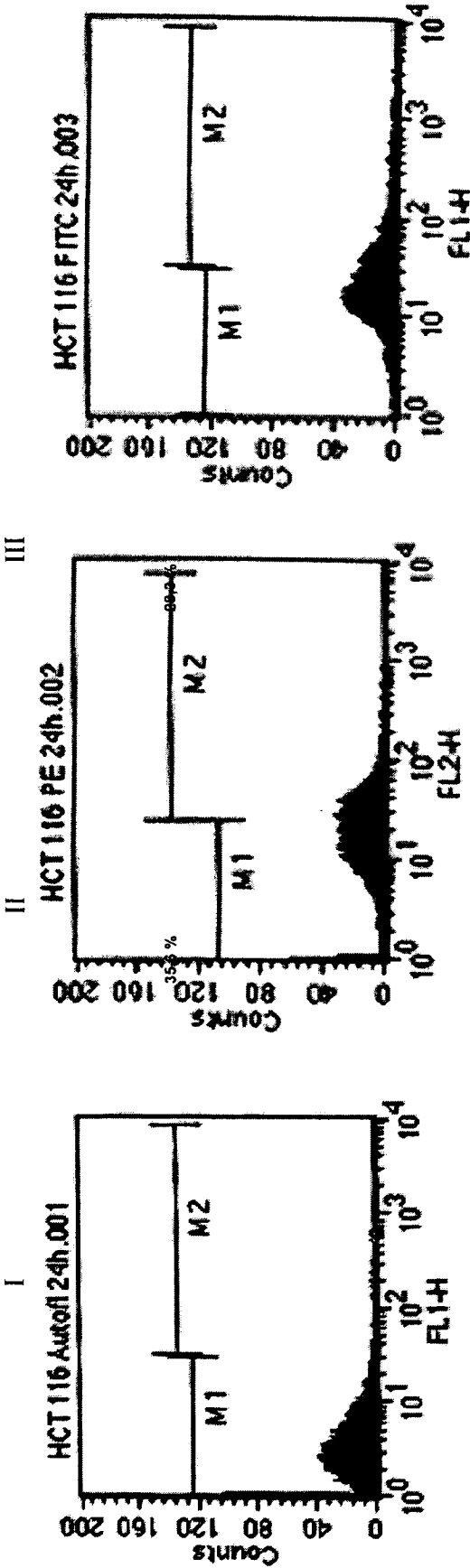


Figure 7



Figure 8

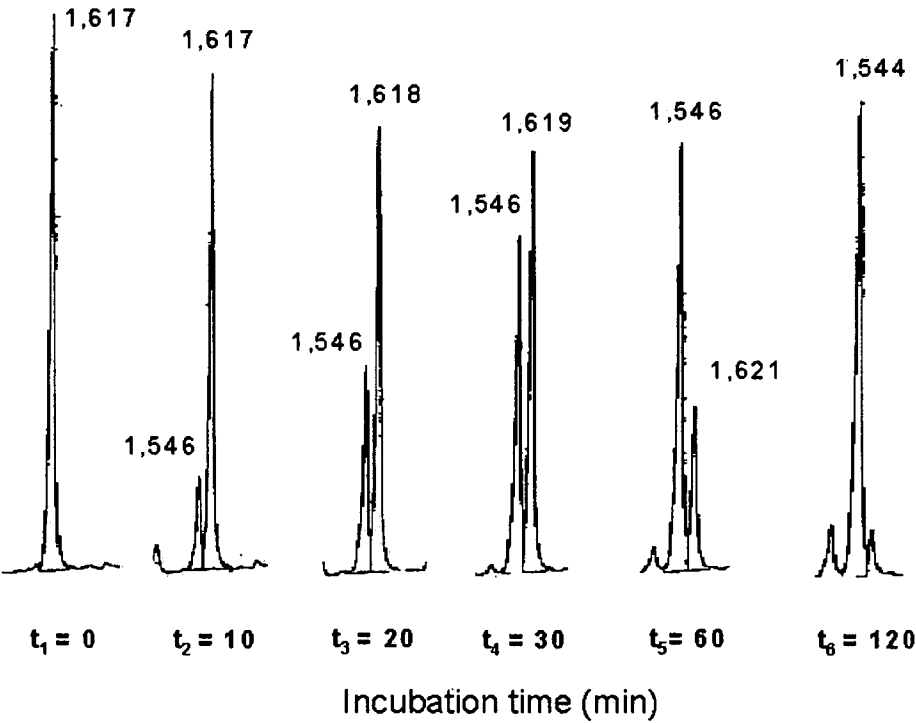
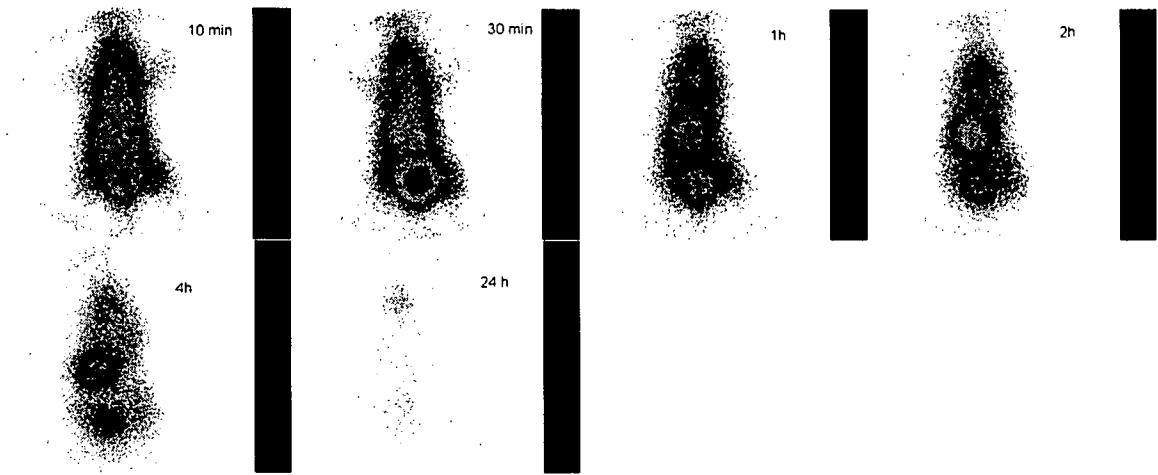


Figure 9



I 125 Ca9P1_M2 7 MBq
SKRC 52



Figure 10

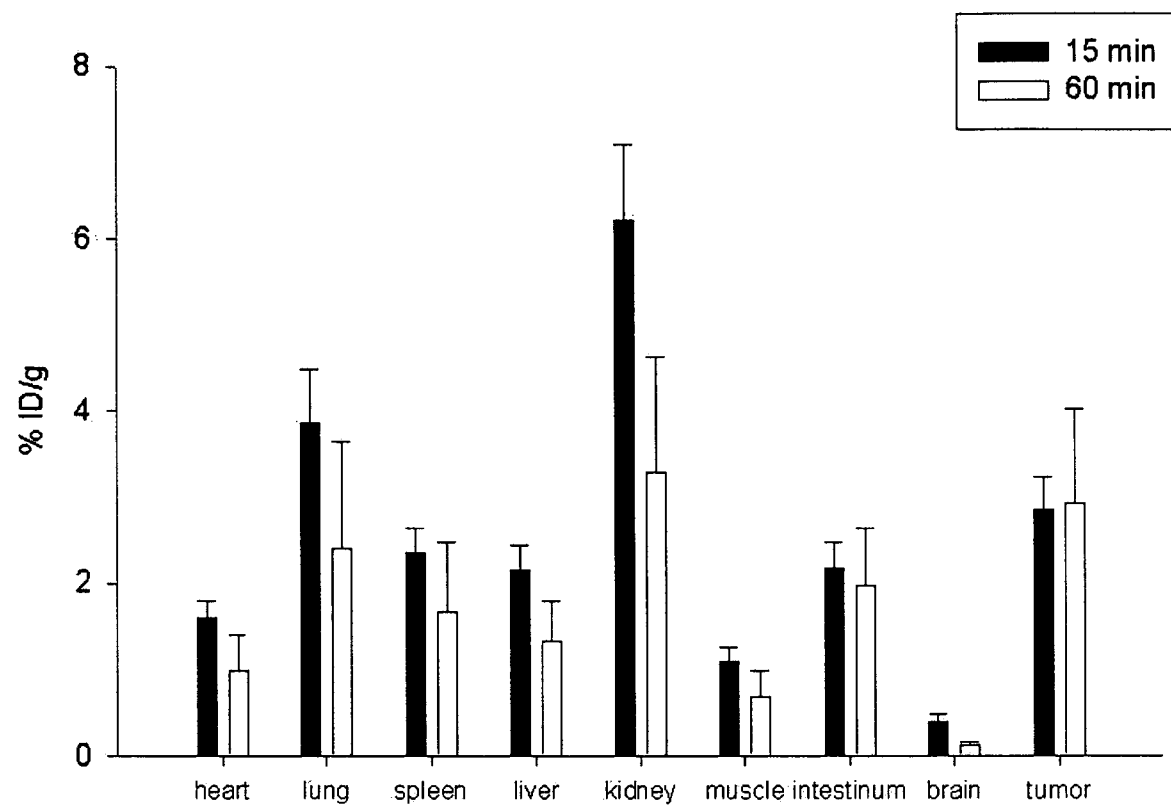


Figure 11

Alanine scanning of CalX-P1

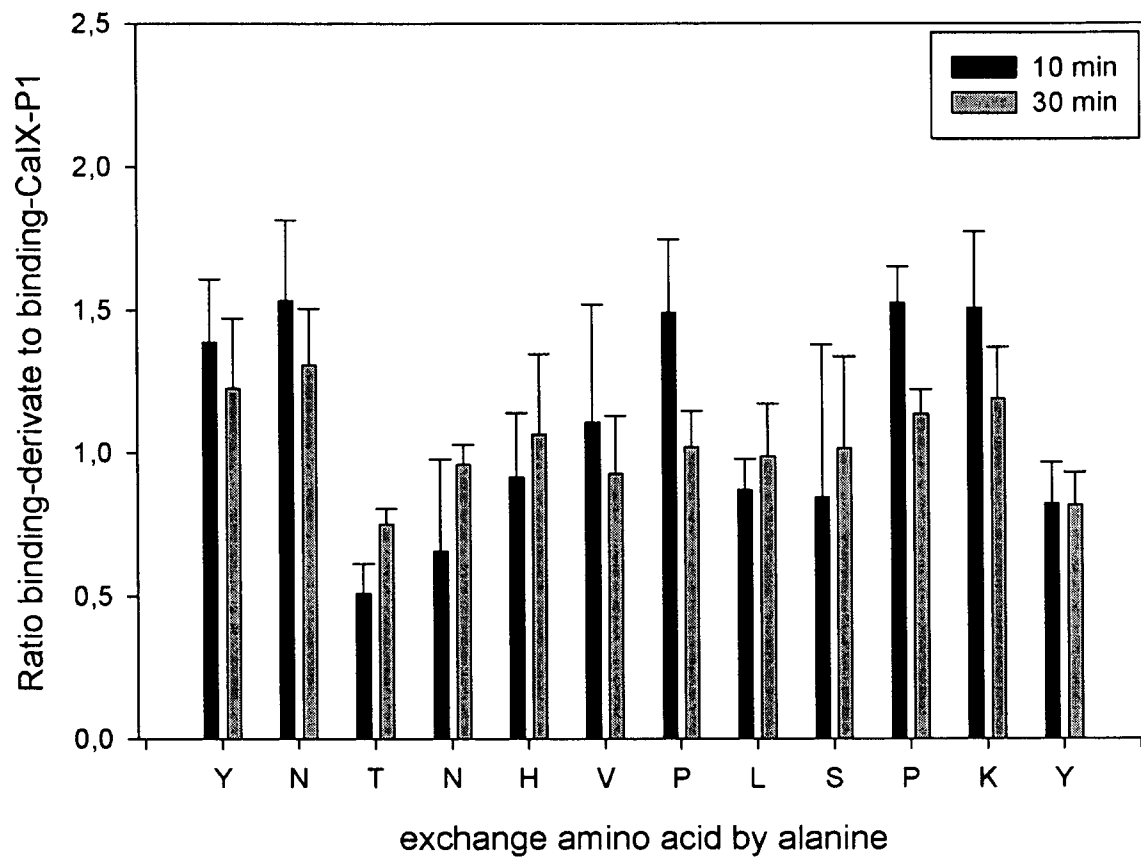


Figure 12

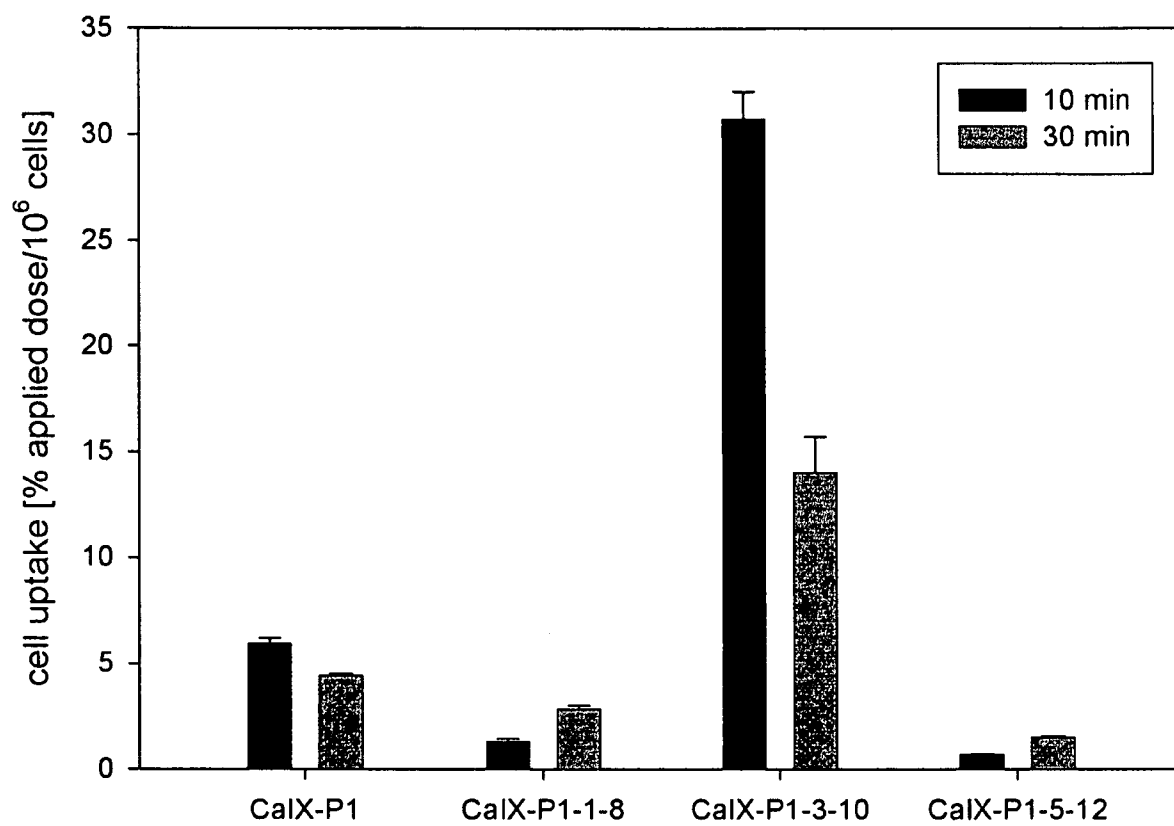


Figure 13

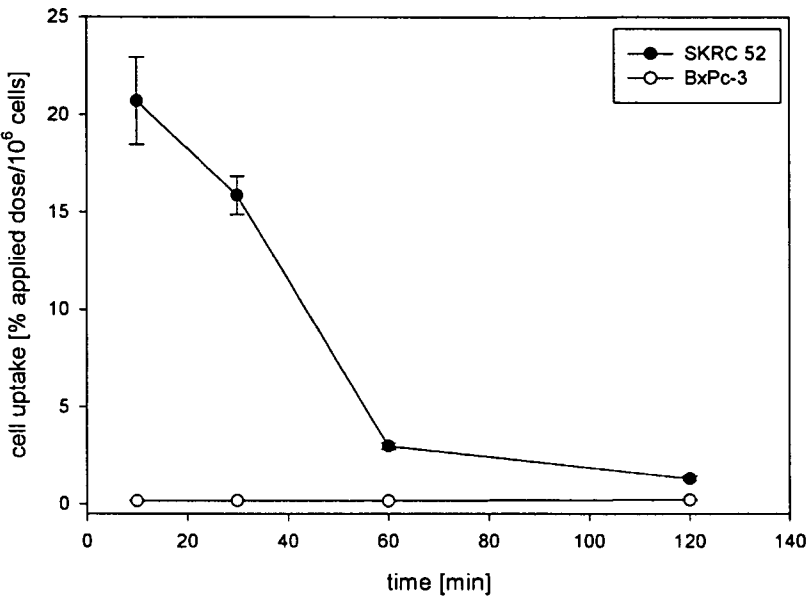


Figure 14

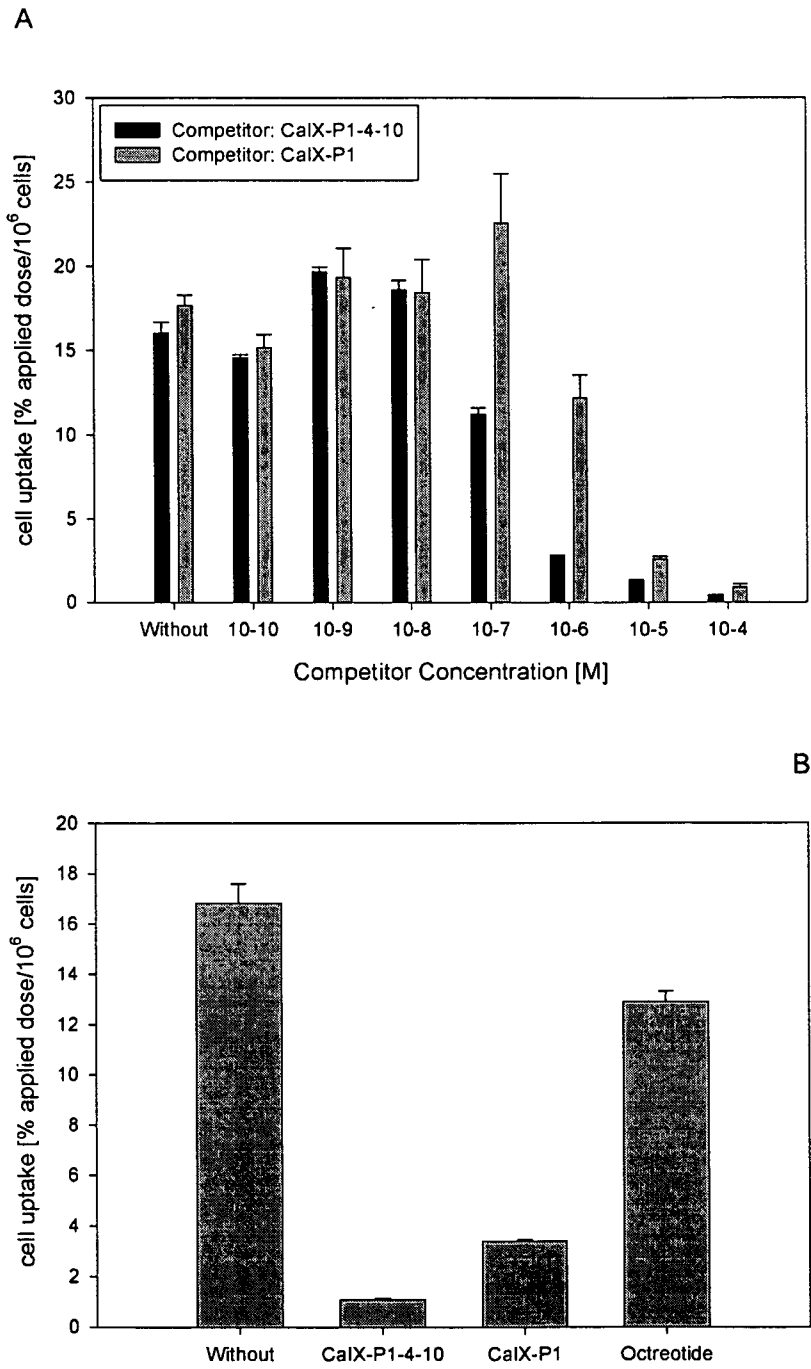


Figure 15

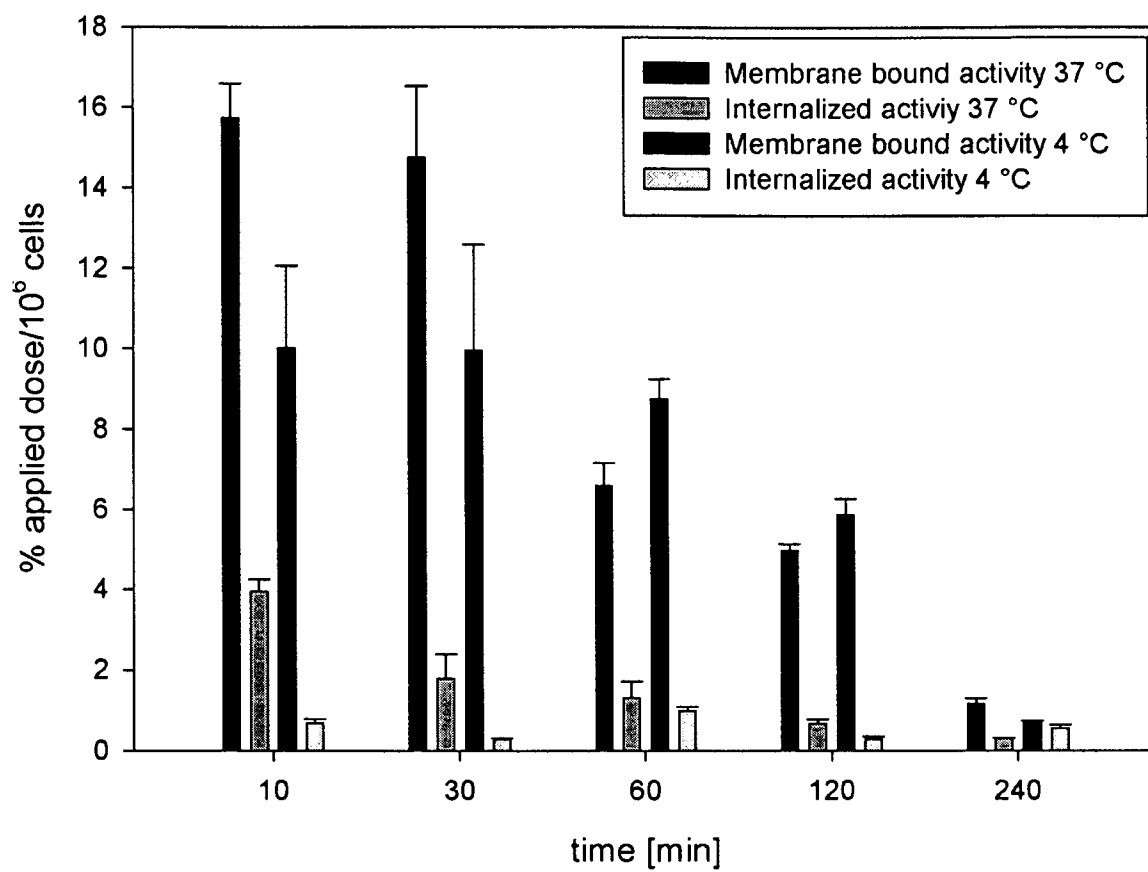


Figure 16

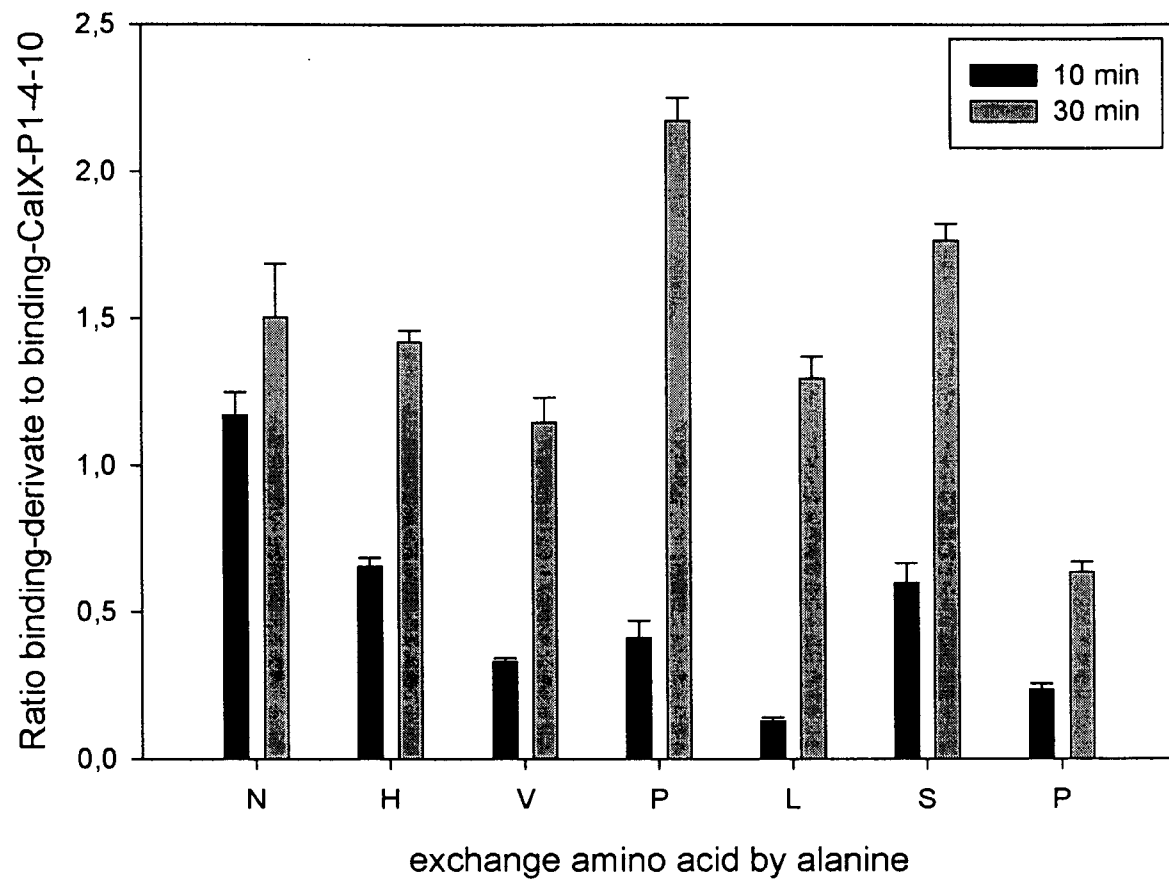


Figure 17

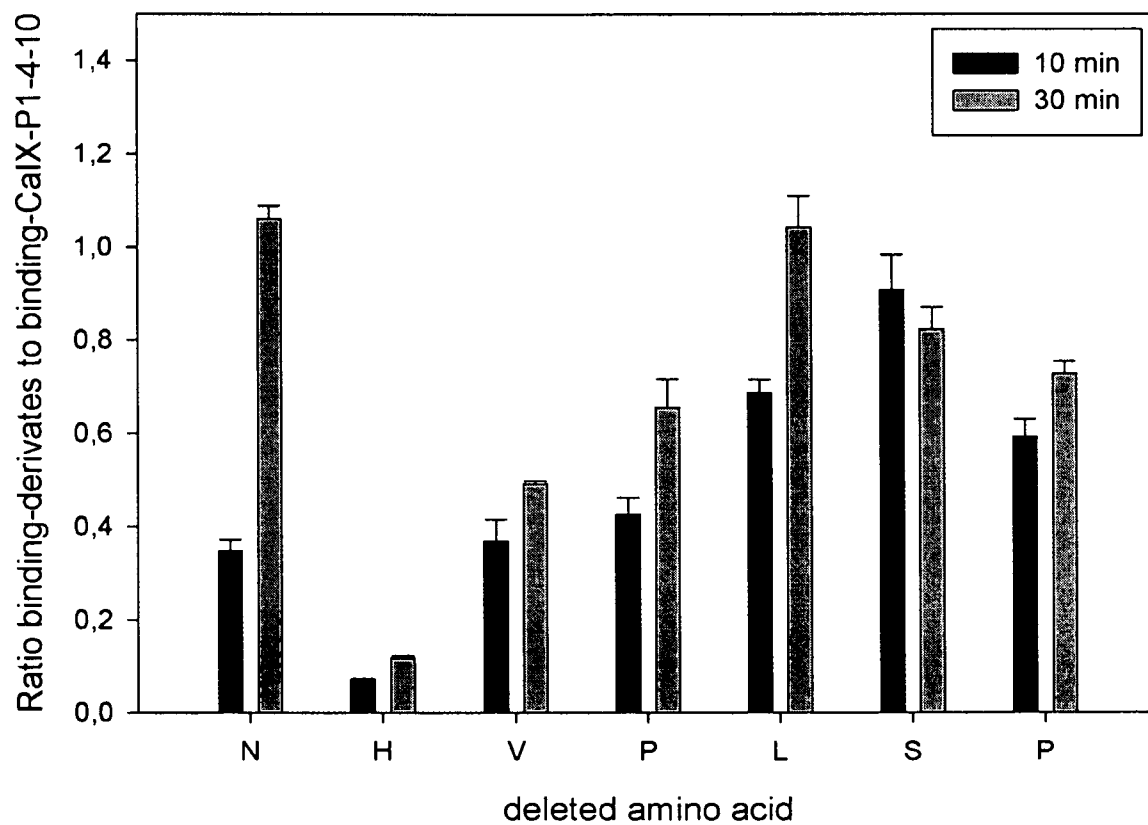


Figure 18

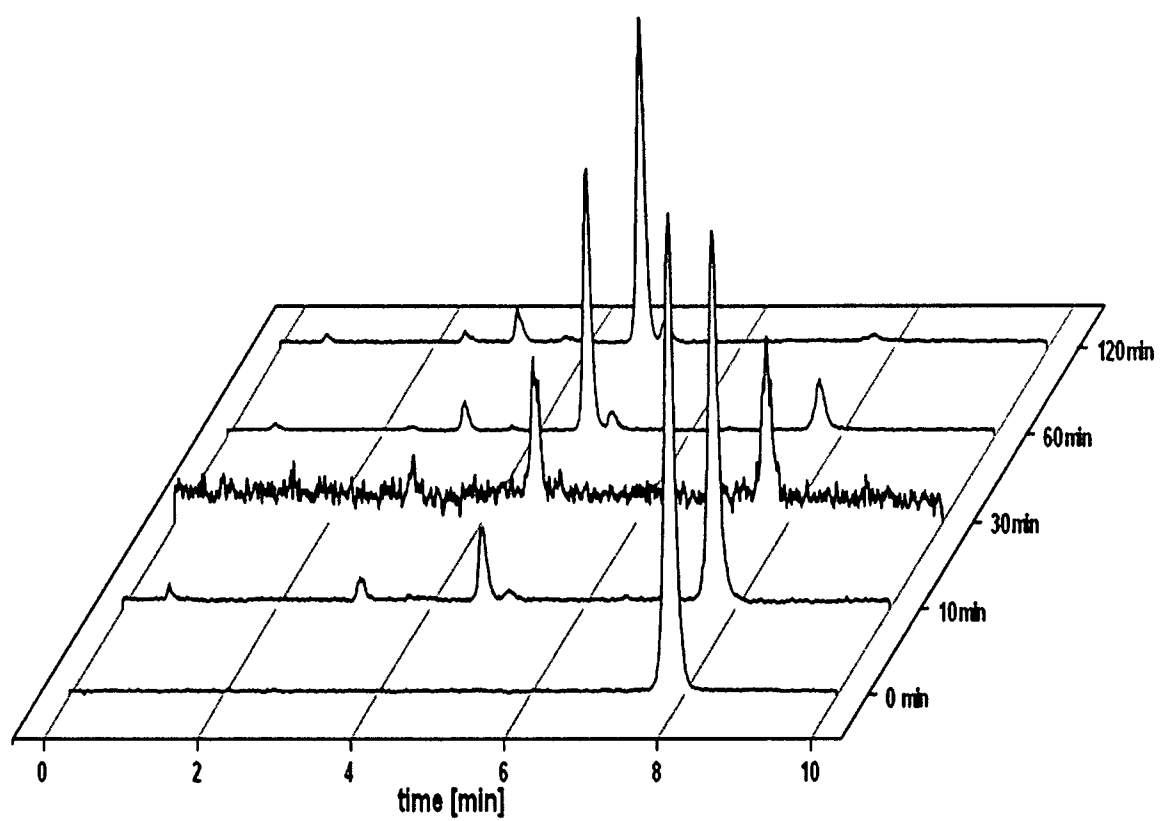


Figure 19

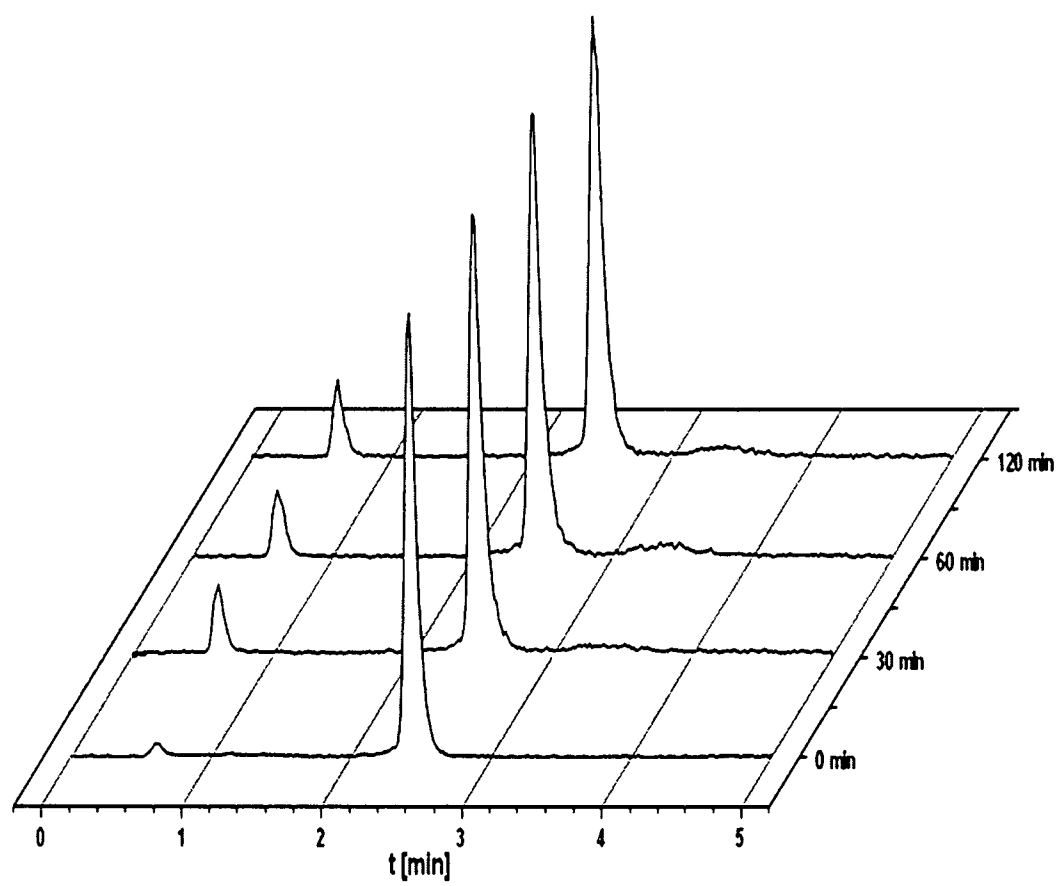


Figure 20

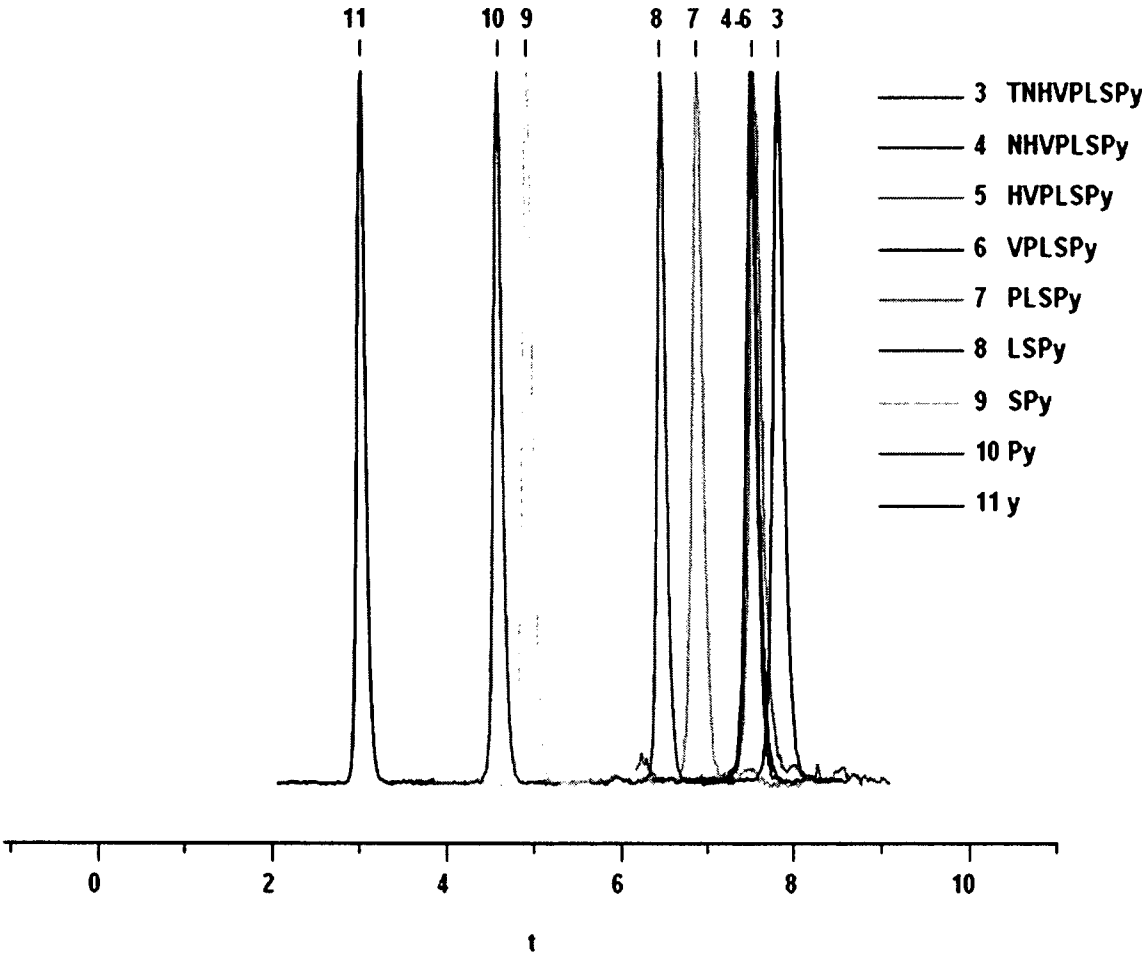


Figure 21

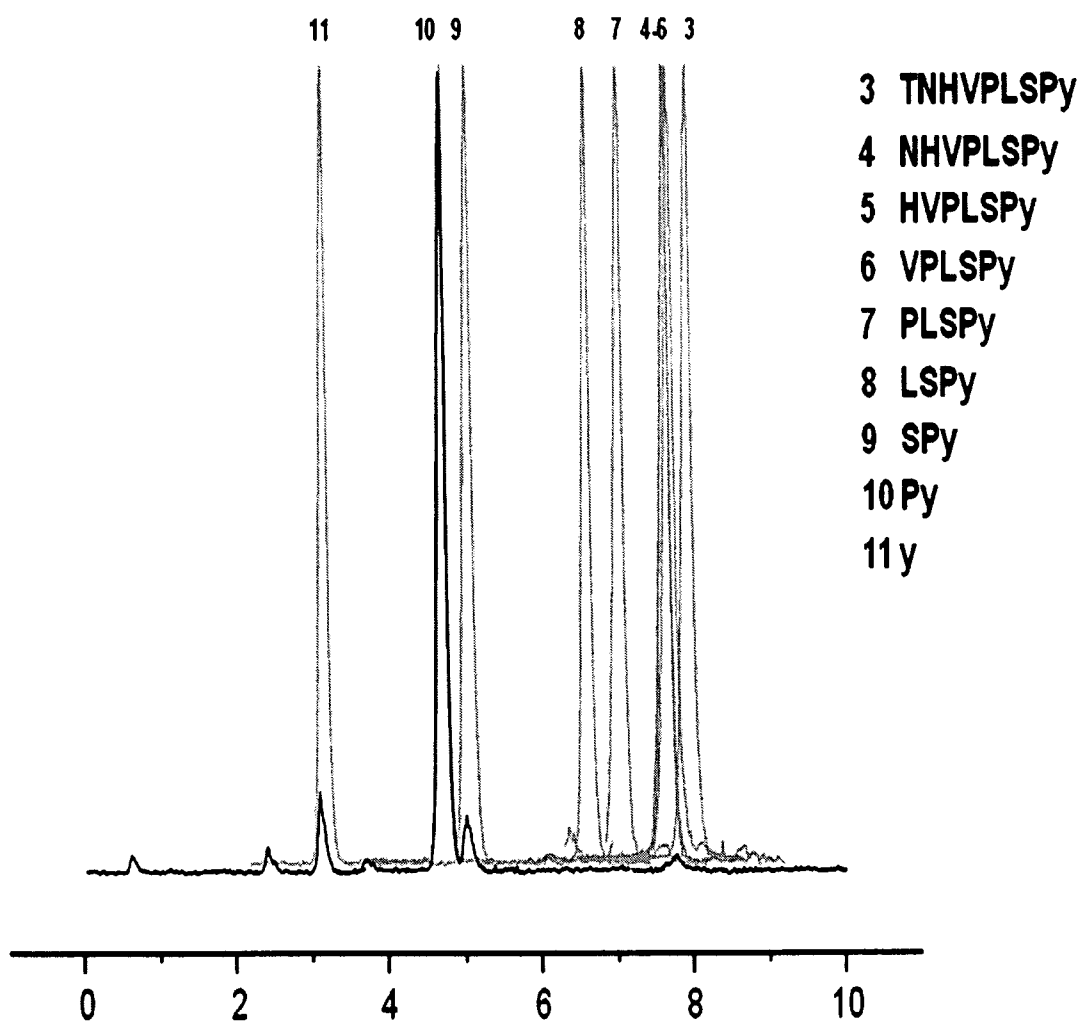


Figure 22

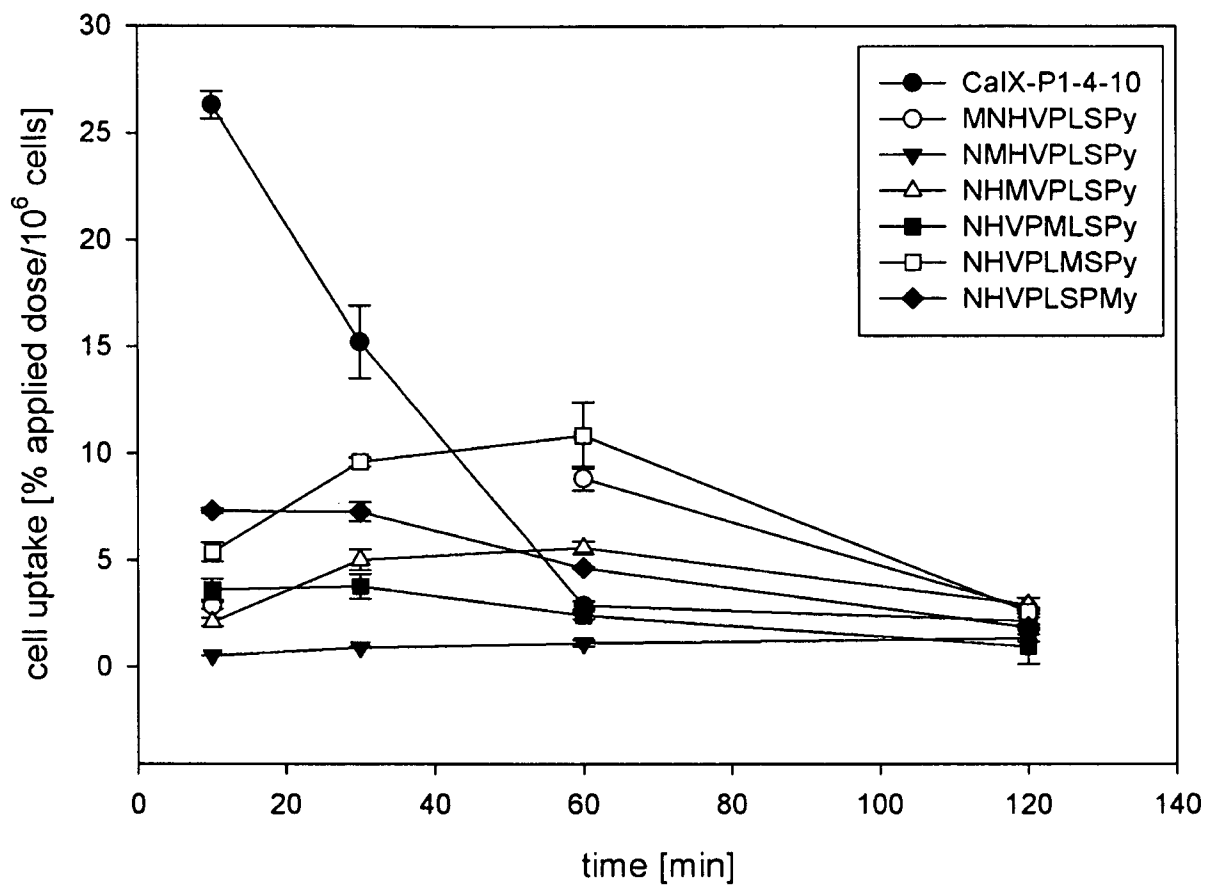


Figure 23

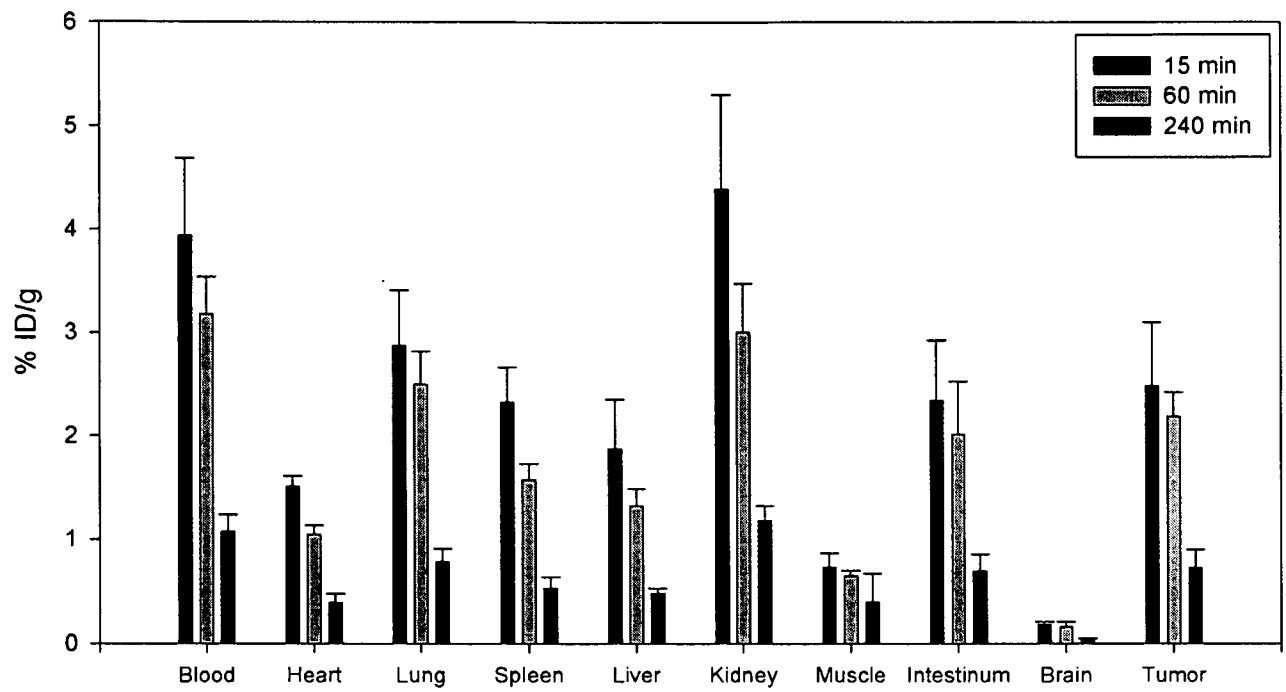


Figure 24

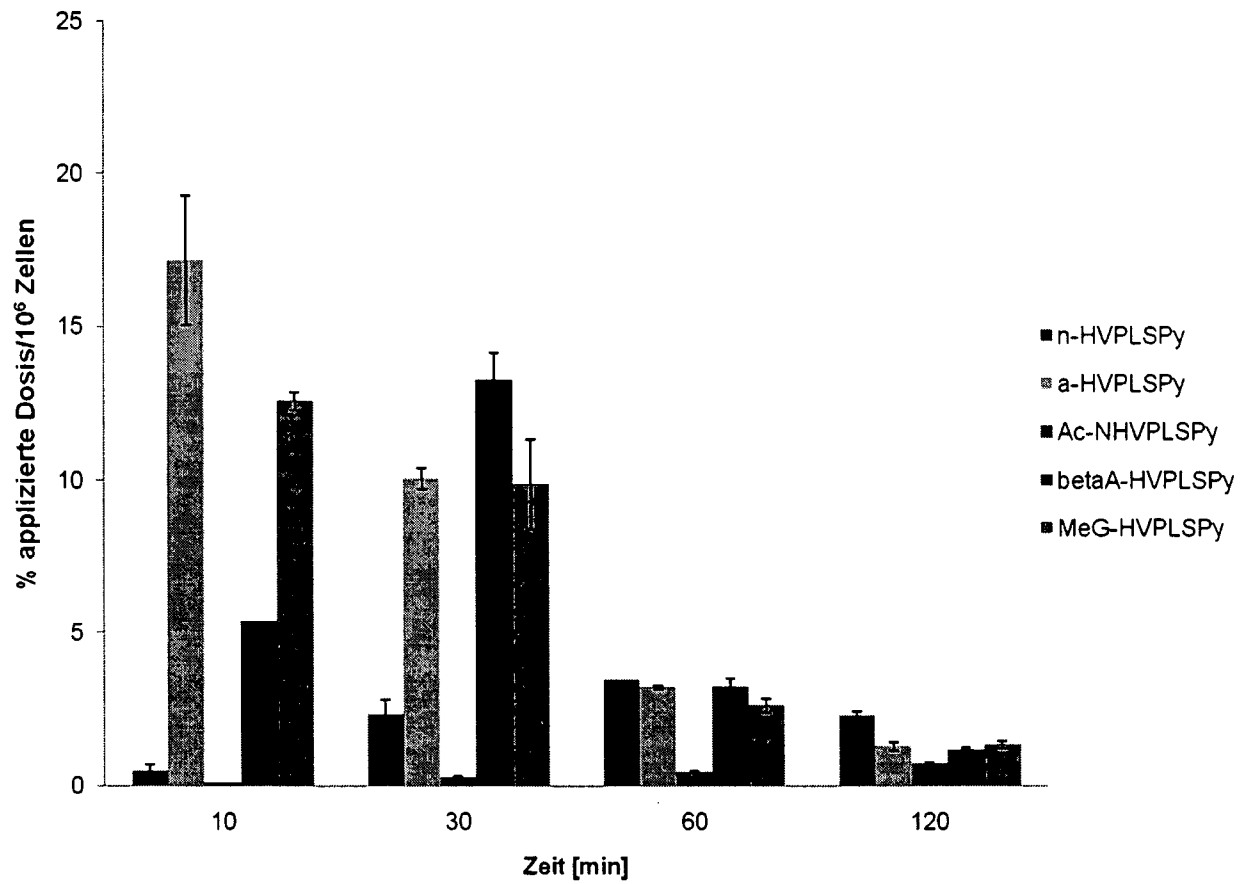
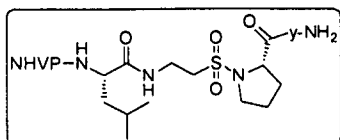
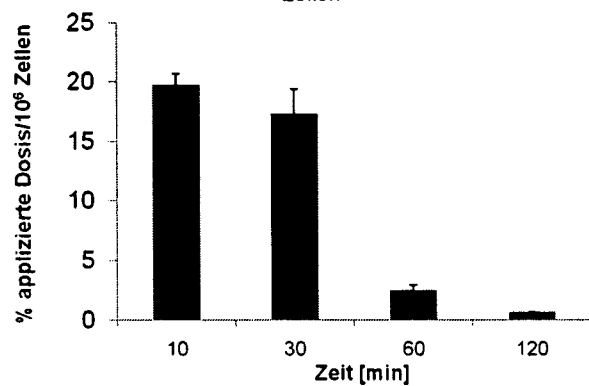
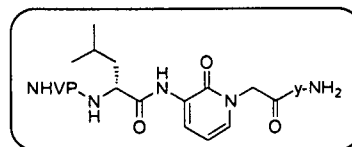


Figure 25

Ala-Substitution via Taurine

Bindungskinetik von 125I-4-10-FN2 auf SK-RC-52 Zellen

*Ala-Pro substitution via Pyridone*

Bindungskinetik von 125I-4-10-FN1 auf SK-RC-52 Zellen

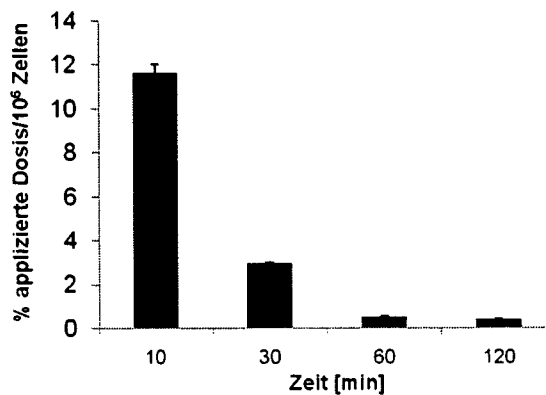


Figure 26

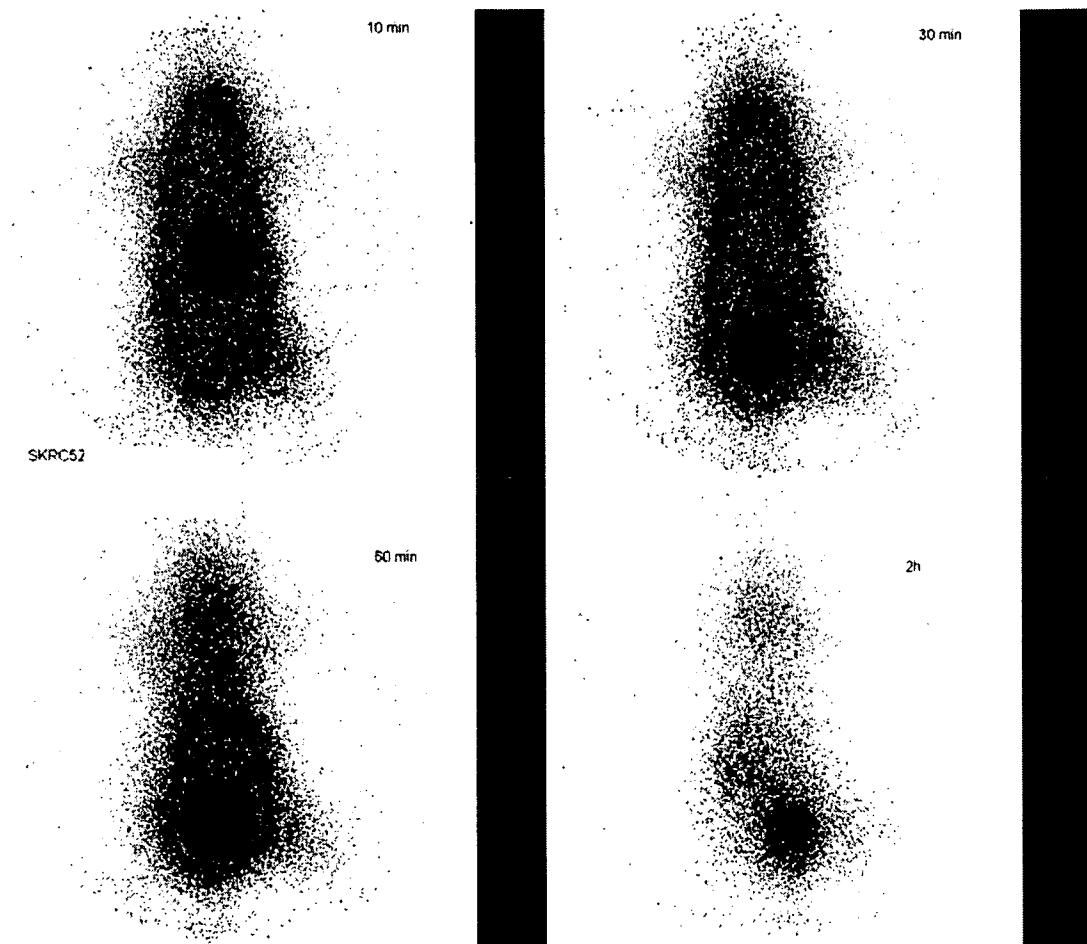


Figure 27

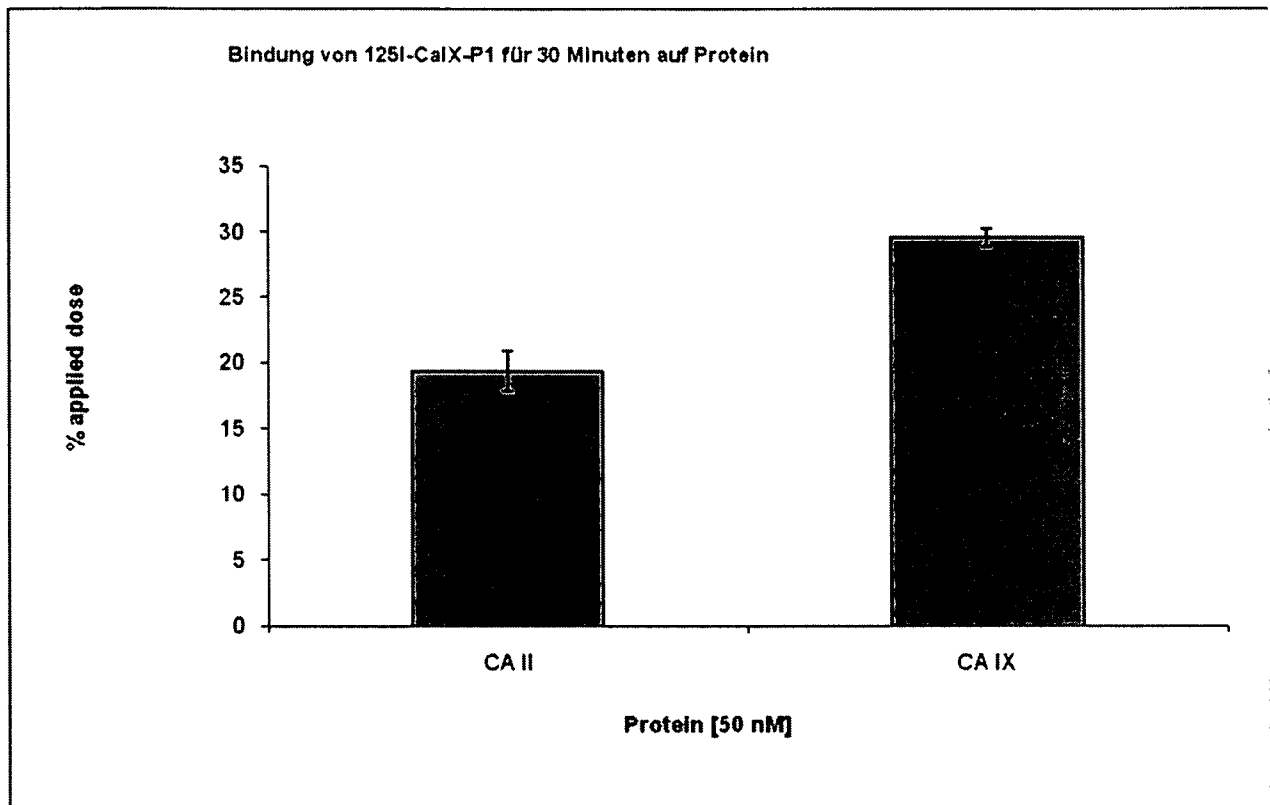


Ca II

Ca IX



Figure 28



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2011/003946

A. CLASSIFICATION OF SUBJECT MATTER
 INV. G01N33/574
 ADD.

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

B. FIELDS SEARCHED

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

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/116468 A2 (DAKO DENMARK AS [DK]; SCHOELLER JOERGEN [DK]; PEDERSEN HENRIK [DK]; BR) 2 October 2008 (2008-10-02) abstract; claims 795,1006,1013,1276,1407; example 13; sequences 10389, 9292 page 58, line 10 - line 13; table 1 page 57, line 5 - line 6 -----	1-18,26, 27
X	WO 2009/024789 A1 (ISIS INNOVATION [GB]; ROWLAND JONES SARAH [GB]; LELIGDOWICZ ALEKSANDRA) 26 February 2009 (2009-02-26) abstract; claim 1; sequence 42 page 16, line 26 - page 17, line 1; claims 1,10,13 ----- -/--	1-11, 14-18, 26,27



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

12 December 2011

Date of mailing of the international search report

13/01/2012

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Mulder, Lonneke

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2011/003946

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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International application No

PCT/EP2011/003946

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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International application No

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