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(54) Title: **RADIOLABELED ANNEXINS**

(57) Abstract: A radiolabeled annexin comprising at least 2 up to 20 histidine residues at its N-terminus is disclosed. At least two of said histidine residues are adjacent or separated by no more than one other amino acid. The radiolabeled annexin is a stable complex with a radionuclide such as technetium 99m. The radiolabeled annexin can be used in a method of imaging cell death in a nucleated cell within a region of a mammalian subject in vivo.



WO 2010/024673 A1

Radiolabeled Annexins

Field of the invention

The invention relates to annexin molecules having binding sites for heavy metals, in particular radioactive metals. The invention further relates to the use of radiolabeled
5 annexin molecules in diagnostic imaging.

Background

Molecular Imaging of the biomarker phosphatidylserine (PS) *in vivo* is important for diagnosis of diseases and assessment of efficacy of therapy. Effective PS-recognising agents are annexin A5 and its variants. Technetium-labeled annexin A5 can be injected
10 into a subject and, subsequently, Single Photon Emission Tomography (SPECT) can be applied to assess the biomarker PS. In order to assess the biomarker PS sensitively and specifically, it is mandatory that the complex between technetium and annexin A5 is stable *in vivo* and is rapidly cleared from the blood circulation.

Current complexes of technetium and annexin A5 are based on chemical bonding of Tc
15 (technetium) to a non-specific site of the annexin molecule. For example, JP2006-316004 discloses a compound obtained by bonding 4'-aminomethyl-N,N'-trimethylene-dibenzohydroxamide to annexin V through a linker, which compound is capable of complexing technetium. WO 98/48699 discloses a complex in which Tc99m is linked to the annexin via succinimidyl hydrazinonicotinate (HYNIC). Such complexes suffer
20 from insufficient stability. Moreover, it is not possible to direct the label at a specific site of the annexins, especially a site which does not interfere with its affinity profile. Jung et al., *J. Nucl. Med.* 2008 49 (Supplement 1):304P, refers to a I-124 labeled recombinant annexin molecule for apoptosis imaging using small animal PET (positron emission tomography).

25 *Summary of the invention*

The present invention provides a novel annexin variant with an extension of its N-terminus carrying histidine residues to allow stable complex formation between a radionuclide such as technetium and annexin. The invention also pertains to such annexins carrying a radionuclide, i.e. to radiolabeled annexins. The invention furthermore
30 provides annexin radiolabeled complexes for use in diagnostics and therapy.

Description of the invention

Thus, the invention pertains to a (radiolabeled) annexin, which comprises a minimum of 2 and a maximum of 20 histidine residues at its N-terminus. In particular, the annexin of the invention comprises a minimum of 3, preferably at least 4 histidine residues at its
5 N-terminus. The maximum number is not very critical, and practically it may be up to 12, or preferably up to 10 or even up to 8 histidine residues at the N-terminus.

Preferably, the histidine residues are contiguous, i.e. without other amino acids in between, or with only one amino acid in between. This particularly applies to two or three histidine residues. Thus, preferred partial sequences at the N-terminus include
10 H-H; H-H-H; H-X-H; H-X-H-H; H-H-X-H; H-X-H-H; or H-X-H-H. An intervening amino acid X, if present at all, is preferably not a large apolar amino acid such as Phe, Tyr, Leu, Ile or Val, or Met or Cys. Preferably, intervening amino acids, if present at all, are selected from Gly, Ala, Ser, Lys, and Arg, and in cases of two or more X, these may be different. Most preferably, the annexin variant of the invention comprises at least 3
15 contiguous histidine residues at its N-terminus, especially at the very terminus.

The term "annexin" refers to any protein capable of binding to phospholipids, especially phosphatidylserine (PS) in a calcium-dependent manner, and members of the so-called annexin family. These substances have the property of binding to negatively charged phospholipids, preferably with a dissociation constant less than 10^{-6} M in the presence
20 of Ca^{2+} ions. The family covers many members; information thereon and on the protein and nucleotide sequences can for example be found on <http://www.structuralchemistry.-org/annexins/seq/search.php>. A sequence alignment of various annexins is also to be found in WO 2007/069895, which is incorporated herein by reference. A preferred example is annexin A5, having the amino acid sequence of SEQ ID No. 7 (Figure 1),
25 but other annexins can also be used for producing and using the annexin variants of the invention. For example, annexins A4 and A8 can also be used. Where reference is made to the amino acid sequence and the positions of annexin A5, this also applies to the other annexins, especially human annexins, by choosing the corresponding position. The corresponding positions are known to the skilled person and can be easily
30 identified, e.g. using the triplet DAE (Asp-Ala-Glu) at positions 19-21 of A5, which is conserved over most annexins, with minor deviations in some annexins, e.g. to DAQ in A4, A9 and A10. The annexins may originate from any species, preferably avian or mammalian, more preferably mammalian, most preferably human.

Wild-type annexins only have a limited number of histidine residues, and never two or more with no or only one other amino acid in between (H-H or H-X-H). For example, wild-type mammalian annexins A5 only contain His residues at positions 97, 204 and 266. Hence, the presence of between 2 and 20 histidine residues with a maximum of one intervening other amino acids is a unique feature of the annexin variants of the present invention.

As used herein, the N-terminus of the annexin variants comprises the sequence upstream of the glutamic acid (E) residue at position 16 of SEQ ID No. 7, or corresponding regions in other annexins. In particular, the annexin variants of the invention have the N-terminal sequences No 1-6, in increasing order of preference:

	X1-ERADAETLRKAMK	(SEQ ID No.1)
	X2-GFDERADAETLRKAMK	(SEQ ID No.2)
	X3-DFPGFDERADAETLRKAMK	(SEQ ID No.3)
	X4-TVTDFPGFDERADAETLRKAMK	(SEQ ID No.4)
15	X5-LRGTVTDFPGFDERADAETLRKAMK	(SEQ ID No.5)
	X6-AQVLRGTVTDFPGFDERADAETLRKAMK	(SEQ ID No.6)

wherein:

each of X1-X6 comprises between 2 and 20 histidine residues separated by no more than one other amino acid residue, and

- 20 X1 represents a sequence of at least 15 amino acids;
- X2 represents a sequence of at least 12 amino acids;
- X3 represents a sequence of at least 9 amino acids;
- X4 represents a sequence of at least 6 amino acids;
- X5 represents a sequence of at least 3 amino acids;
- 25 X6 represents a sequence of at least 2 amino acids.

Herein, the part starting with the first E (Glu) of SEQ ID No. 1 (= position 16 in SEQ ID No. 7) can be different in other annexins. For example, it is AMEDAQTLRKAMK in human A4, and PDPDAETLYKAMK in human A8. Exchanges of single amino acids in that part corresponding to other annexins are allowed, for example an exchange of Glu (E) at position 16 by Ala (A) or Pro (P).

In a special embodiment of the invention, the annexin variant carries one or more, preferably one up to three cysteine residues, most preferably just one cysteine residue,

at the concave side of the annexin molecule. The concave side of the annexin molecule corresponds to the underlined amino acids in the sequence of Figure 1 (SEQ ID. No. 7). Thus, the cysteine residue is preferably present at one of the positions 1-19, 24, 28, 46-64, 86-89, 118-135, 150, 157-170, 202-219, 245-248, and 280-294 of annexin A5 or
5 corresponding positions of other annexins.

It is at the same time preferred, that the annexin variant does not carry cysteine residues at the convex side of the molecule. This means that e.g. in annexin A5, the (only) cysteine residue at position 315 is preferably substituted by another amino acid, such as Ser, Ala or Val. Details about the positions of the cysteine molecule and the manner of
10 introducing them can be found in WO 2006/003488, which is incorporated herein by reference.

A specifically preferred polyhistidine annexin variant of the invention is the annexin variant having the amino acid sequence depicted in Figure 2 (SEQ ID No.8), or a variant which contains 3, 4, 5, 7, 8, 9 or 10 N-terminal His residues instead of the 6 as
15 depicted. Further preferred variants are those having the amino acid sequence of Fig. 2, wherein one of the amino acids at positions 7-25, 30, 34, 52-70, 92-95, 124-141, 156, 163-176, 208-225, 251-254, and 286-300. Other preferred variant are those, which contains a Cys residue at one of the amino acids at the positions mentioned here above (7-25 etc.) of Fig. 2, wherein further the cysteine residue at position 321 is substituted
20 by another amino acid, especially Ser, Thr, Ala or Val.

Further amino acid substitutions may be present in the annexin variants and radio-labeled annexins of the invention. For example, an amino acid substitution at one or more of the positions 16-29, 59-74, 88-102, 135-145, 156-169, 202-231, 259-266 and 305-317 of annexin A5 (or the positions which are each six higher in the poly-His
25 annexin A5 variant of Fig. 2) may be substituted so as to inhibit internalisation of the annexin variant into the target cell, if so desired. Such substitution is preferably a substitution of a polar amino acid by an apolar one, as described in WO 2007/069895. Other amino acid substitutions are also allowed, provided that they do not significantly hamper the binding of the annexin to phosphatidylserine. This provision is deemed to be
30 met if the substitution is a substitution by an amino acid present at the same position in another annexin type, especially if the other type is annexin A4 of A8, or in an annexin of the same type from another species.

The desired amino acid additions or substitutions can be performed by recombinant techniques well-known in the art and illustrated in the examples below. The histidine residues can be introduced into the DNA encoding the annexin variant of the invention by substitution of two or more codons encoding N-terminal amino acids by histidine encoding codons (CAU/CAT or CAC), for example substitution of codons for N-terminal AQV (Ala-Gln-Val) of annexin A5 by three histidine codons.

Alternatively, or additionally, the annexin-encoding gene can be extended at its N-terminus with two or more, e.g. 3, 4, 5 or 6, histidine codons. Extension of annexin molecules as such is known in the art. For example, WO2005/086955 discloses a human annexin V homodimer with a 6-His tag, and Tabata et al., *J. Biosc. Bioeng.* 1001 (2006) 190-197, describes a 6-His-tagged annexin A2. Polyhistidine derivatives can be produced by recombinant methods known in the art. They are conventionally used for purification purposes and often contain additional amino acids facilitating removal of the polyhistidine tag by specific proteolysis. In contrast, the polyhistidine variants are not to be proteolysed, and preferably do not contain such additional specific proteolysis sites. Vectors for producing polyhistidine tags are commercially available, e.g. from Qiagen, Venlo, NL.

If desired, a codon for any one of the underlined amino acids in Fig. 1, for example the codon encoding Phe at position 11, can be substituted by a cysteine codon and/or the Cys codon at position 315 can be substituted by a codon for another amino acid, for example Ser. The modified annexin gene can then be expressed in a suitable host to produce the desired annexin variant of the invention.

The cysteine residue can be used to couple pharmacological agents or further diagnostic agents to the annexin. A linker such as N-succinimidyl 3-(2-pyridyldithio)propionate, N-succinimidyl maleimidoacetate, N-succinimidyl 3-maleimidopropionate, pyridyl, maleimide-containing groups, halogen-containing groups, can be used to couple the agent to the Cys residue. The pharmacological agent can e.g. be selected from a toxin, an enzyme, a lipid, a carbohydrate, an immunoglobulin or a fragment thereof, an immunoconjugate, a chemotherapeutic compound, a photosensitizer, a radionuclide, a cell death inducing agent, a cell death inhibiting agent, a fibrinolytic compound. The (further) diagnostic compound can be selected from e.g. a fluorescent group, a contrast agent, a photosensitiser, an ultrasound agent etc. Further details are described in WO 2006/003488.

- The presence of at least two histidine residues allows the annexin variant of the invention to bind a radionuclide, especially a metal radionuclide. Herein, the histidine residues act as multifunctional chelators for the (metal) radionuclide. Thus, the invention also pertains to a radiolabeled annexin, which is a complex of a histidine-substituted annexin variant as described above and a radionuclide. The radionuclide is preferably biocompatible and is preferably selected from Gallium 67, Gallium 68, Indium 111, Technetium 99m, Rhenium 188, Copper 64 and Tin 117m. Most preferably, the radionuclide in the radiolabeled annexin according to the invention is Tc^{99m} or Re^{188} , most especially Tc^{99m} .
- 10 The radionuclides can be coupled to the histidine-containing annexin variants in a manner known in the art. For example, the variant can be contacted with a tricarbonyl complex of the radionuclide such as technetium or rhenium to produce a radionuclide-linked annexin. For ^{99m}Tc tricarbonyl, a reagent kit is commercially available (Mallinckrodt, Petten, NL).
- 15 The radiolabeled annexin can be used in a method of imaging cell death in a nucleated cell within a region of a mammalian subject *in vivo*. Such a method can comprise:
- (a) administering said radiolabeled annexin to the subject,
 - (b) positioning the subject within the detection field of a radiation detector device, and
 - (c) measuring radiation emission from the radionuclide in the subject, with the radiation
- 20 detector device, to construct an image of radiation emission, wherein said image is a representation of cell death in said nucleated cell of said mammalian subject.
- In step (a) of this method, a radiolabeled annexin (e.g. technetium 99m-labeled annexin V) is administered to the subject using standard protocols. A period of time is then
- 25 allowed to achieve localisation of the radiolabeled annexin in the subject. Then, in optional step (b), the subject is placed within the detection field of a radiation detector device. The subject is maintained in a substantially immobilised condition while radiation from the radionuclide is measured using the radiation detector device (step c). The measured data are then processed to an image of the radiation emission. The image
- 30 thus obtained can be used to provide the attending clinician with a map or a localisation of areas of cell death in the mammalian subject, or in the region of the mammalian subject that is being analysed.

The radiolabeled annexin may be administered in several ways. In a preferred embodiment, the radiolabeled annexin is administered intravenously. Alternatively, it can be administered intraperitoneally. A further option is to administer the labeled annexin intrathecally. Also, the radiolabeled annexin can be administered intrapleurally.

- 5 Further methods of administration comprise intralymphatic administration, or, alternatively, intramuscular administration.

The dosage of radiolabeled annexin to be administered depends on the radionuclide used, on the tissue of organ which is targeted, and on the conditions of the diagnosis and the subject. Preferably, the radiolabeled annexin is administered in an amount which
10 results in a dose of between about 3 and about 30 mCi. Technetium 99m can be administered to adult humans at doses up to about 20 mCi. The preferred dose for a single Tc99m administration is between about 5 and 20 mCi, preferably between 7.5 and 15 mCi. The amounts of other radionuclides can be determined accordingly.

The amount of radiolabeled annexin to be administered (calculated on the basis of the
15 amount of annexin as such) is preferably at least 0.3 µg/kg body weight, and below 300 µg/kg. Typically, the amount is less than about 100 µg/kg. Preferably, the amount is between 0.5 and 20 µg/kg, most preferably between 1 and 20 µg/kg.

After the radiolabeled annexin is administered, it is allowed to be localised to the target tissue or organ. When an equilibrium or a quasi-equilibrium between localised and non-
20 localised or unbound annexin is achieved, usually between 10 and 240 minutes, especially between 20 and 120 minutes, the measurement can start. If necessary, the state of localisation as a function of time may be followed by imaging the radiation signal from the labeled annexin. Where the radionuclide is technetium 99m, the radiation will be γ emission. WO 98/48699, which is incorporated herein by reference,
25 gives further information and examples of imaging cell death in a mammalian subject.

Preferred uses for radiolabeled annexins include the detection of inappropriate apoptosis in diseased states, where it is undesired, e.g. immune disorders such as Lupus, transplant rejection, or in cells subject to ischemia; and the detection of insufficient apoptosis where it is desired, e.g., tumours or cells infected with a virus. In particular,
30 the cell death is caused by necrosis.

The radiolabeled annexin can be used in a variety of clinical settings in which apoptotic and/or necrotic cell death is to be monitored, such as organ and bone marrow transplant

rejection or injury, infectious and non-infectious inflammatory diseases, autoimmune disease, cerebral and myocardial infarction and ischemia, cardiomyopathies, atherosclerotic conditions, neural and neuromuscular degenerative diseases, sickle cell disease, β -thalassemia, cancer therapy, AIDS, myelodysplastic syndromes, and toxin-induced liver disease, etc. Radiolabeled annexins are also useful as a clinical research tool to study the normal immune system, embryological development, and immune tolerance and allergy.

Radiolabeled annexin V can be used, for example, to image and quantify apoptotic cell death in normal and malignant tissues undergoing treatment. Monitoring apoptosis with serial imaging studies using radiolabeled annexin can be used for the rapid testing and development of new drugs and therapies in a variety of diseases. In addition, the methods may be used to monitor the progress of treatment, monitor the progress of disease, or both. Further, they may be used to aid in early detection of certain diseases.

The region of the mammalian subject where cell death is to be imaged *in vivo*, may be any part, tissue or organ of the subject. In particular, said region is in an organ of said subject or a portion thereof. In a preferred embodiment of the invention, the region is in the head of said subject or a portion thereof. In another preferred embodiment, said region is in the heart of said subject or a portion thereof. In a further specific embodiment, said region is in the liver of said subject or a portion thereof. The region wherein (desired) cell death is to be imaged can especially be in a tumour in the mammalian subject or a portion thereof. Also, the region (of undesired) cell death can be a transplant in the subject or a portion thereof. Further, said region can be in an ischemic site in said subject or a portion thereof.

The imaging can be performed using methods and equipment known in the art. For example, the radiation detector device is a positron emission detector device, in case of use of e.g. Cu^{64} as a radionuclide. For gamma-radiating radionuclides, such as $\text{Tc}^{99\text{m}}$, $\text{Sn}^{117\text{m}}$ and Re^{188} , gamma ray imaging devices can be used. The signals can be detected, enhanced, processed, using standard techniques. For example, single photon emission computed tomography (SPECT) may be used with the types gamma-emitting radionuclides described herein, e.g., $\text{Tc}^{99\text{m}}$.

Example 1: *Annexin A5 with an N-terminal extension of 6 histidine residues binds to phosphatidylserine*

The 5'-end of the cDNA of annexin A5 was extended with 6 nucleotide triplets each encoding histidine (CAT or CAC) using standard molecular cloning techniques known by a person skilled in the art. The cDNA of annexin A5 can either encode human wild-type annexin A5 or a variant thereof.

- 5 The extended cDNA is cloned into a prokaryotic expression vector suitable for production of the histidine-extended annexin A5 (His-anxA5) by bacteria. Other expression systems such as eukaryotic expression systems can also be used to produce the His-anxA5. The cDNA is then cloned into an expression vector suitable for eukaryotic expression systems.
- 10 The bacterially produced His-anxA5 is purified from the other bacterial constituents by employing the metal-binding property of the histidine residues. The mixture of proteins is adjusted to 5-20 mM of imidazole and this mixture is applied to nickel or cobalt affinity chromatography. After washing the bound His-anxA5 is eluted from the column by a gradient of 50 - 1000 mM imidazole. The eluted His-anxA5 has a high purity
- 15 (>90% pure) and can be used for biological analysis.
- The phosphatidylserine-binding properties of His-anxA5 are comparable to wild-type annexin A5 as analysed by ellipsometry (Andree et al. JBC 1990) and flow cytometry of apoptotic cells (Van Genderen et al. Nature Prot. 2006, 363). Hence, extension of the N-terminal tail of annexin A5 with 6 histidine residues does not alter the biological
- 20 property to bind to phosphatidylserine.

Example 2: Radiolabeling of histidine extended annexin A5

- His-anxA5 was labeled with the radionuclide ^{99m}Techetium using the commercially available Isolink kit and following the instructions of the manufacturer of Isolink (Mallinckrodt, Petten, the Netherlands). Analysis by size-exclusion chromatography
- 25 (BioSep-SEC-S3000) showed that both the radiochemical purity and radiochemical yield of ^{99m}Techetium-labeled His-anxA5 was more than 95%. The high yield and purity avoid a necessity of purification steps following radiolabeling.

Example 3: Stability of radiolabeled histidine extended annexin A5

- His-anxA5 was radiolabeled with ^{99m}Techetium as described by example 2.
- 30 ^{99m}Techetium labeled His-anxA5 was incubated in blood plasma during 1-24 hours at 37°C. Subsequent analysis revealed that ^{99m}Techetium-labeled His-anxA5 is stable in blood plasma and neither decomposes nor causes transchelation of plasma proteins, e.g.

transfers the ^{99m}Tc isotope to other plasma proteins. This stability is extremely important to its use as a nuclear agent in nuclear imaging technologies.

Example 4: *Binding of ^{99m}Tc labeled His-anxA5 to apoptotic cells in vitro*

His-anxA5 was radiolabeled with ^{99m}Tc as described by example 2. T-lymphoma cells (Jurkat cells) were cultured in vitro and triggered to execute apoptosis and expose phosphatidylserine. The treated Jurkat cells were mixed with ^{99m}Tc labeled His-anxA5, incubated for 5-30 minutes and centrifuged to a pellet. The supernatant was separated from the cells. Part of the cells were resuspended in calcium containing buffer (0.5-10 mM CaCl_2) and measured for radioactivity. Part of the cells was resuspended in EDTA containing buffer (1-10 mM EDTA) and recentrifuged. The resulting supernatant was measured for radioactivity. These analyses demonstrated that ^{99m}Tc labeled His-anxA5 has biological properties to bind in a calcium-dependent manner to apoptotic cells and phosphatidylserine that are comparable to annexin A5.

Example 5: *Nuclear imaging of apoptosis using ^{99m}Tc labeled His-anxA5 in a mouse model of liver apoptosis*

Mice were treated with intravenous injection of anti-Fas antibody or cycloheximide in order to induce liver apoptosis. 30 -120 minutes following injection of the apoptosis inducer, ^{99m}Tc labeled His-anxA5 was injected intravenously. ^{99m}Tc labeled His-anxA5 was prepared as described by example 2. 30 - 240 minutes following injection of ^{99m}Tc labeled His-anxA5 SPECT analysis was performed. Thereafter the liver was taken out for analysis by autoradiography and immunohistochemical staining of caspase 3. The results demonstrate that it is possible to image non-invasively cell death using ^{99m}Tc labeled His-anxA5.

Example 6: *Nuclear imaging of apoptosis using ^{99m}Tc labeled His-anxA5 in a mouse model of cancer.*

Mice were injected in the flank with Daudi cells or Granta519 cells. After 4-6 weeks visible tumours had developed. The mice were injected intravenously with ^{99m}Tc -labeled His-anxA5 was injected intravenously that was prepared as described by example 2. The uptake of ^{99m}Tc labeled His-anxA5 by the tumour was measured non-invasively by SPECT. The mice were then treated with a cytostatic such as doxorubicin and cyclophosphamide. 24 - 72 hours following treatment the mice were injected

intravenously ^{99m}Tc labeled His-anxA5. Uptake by tumour was assessed non-invasively by SPECT. The results demonstrated that the efficacy of anti-tumour therapy can be assessed non-invasively using ^{99m}Tc labeled His-anxA5.

Claims:

1. A radiolabeled annexin, in which an annexin molecule comprises a minimum of 2 and a maximum of 20 histidine residues at its N-terminus, at least two of said histidine residues being adjacent or separated by no more than one other amino acid and being bound to a radionuclide.
2. A radiolabeled annexin according to claim 1, in which the radionuclide is selected from the group consisting of Gallium 67, Gallium 68, Indium 111, Technetium 99m, Rhenium 188, Copper 64 and Tin 117m.
3. A radiolabeled annexin according to claim 2, in which the radionuclide is ^{99m}Technetium.
4. A radiolabeled annexin according to any one of claims 1-3, which comprises from 3 up to 10 histidine residues at its N-terminus.
5. A radiolabeled annexin according to any one of claims 1-4, in which said histidine residues are located upstream of the Glu residue at position 16 of SEQ ID No. 7.
6. A radiolabeled annexin according to any one of claims 1-5, which has the N-terminal sequence:

X4-TVTD FPGFDERADAETLRKAMK (SEQ ID No.4)

wherein X4 comprises said histidine residues and represents a sequence of at least 6 amino acids.
7. A radiolabeled annexin according to any one of claims 1-7, in which one of any of the amino acids at positions 1-19, 24, 28, 46-64, 86-89, 118-135, 150, 157-170, 202-219, 245-248, and 280-294 of SEQ ID No. 7 is replaced by a cysteine residue.
8. A radiolabeled annexin according claim 7, in which said cysteine residue is coupled to a pharmacological agent.
9. An annexin variant having the amino acid sequence of SEQ ID No. 8.
10. An annexin variant having the amino acid sequence of SEQ ID No. 8, in which one of the amino acids at positions 7-25, 30, 34, 52-70, 92-95, 124-141, 156, 163-176, 208-225, 251-254 and 286-300 is substituted by a cysteine residue and the cysteine residue at position 321 is substituted by another amino acid, and in which the substituted cysteine residue at the one of the positions 7-25, 30, 34, 52-70, 92-

95, 124-141, 156, 163-176, 208-225, 251-254 and 286-300 is optionally coupled to a pharmaceutical agent.

11. A radiolabeled annexin according to any one of claims 1-8, for use in imaging cell death in a nucleated cell within a region of a mammalian subject *in vivo*.
12. A radiolabeled annexin according to claim 11, in which said use comprises:
 - (a) administering said radiolabeled annexin to the subject,
 - (b) if applicable, positioning the subject within the detection field of a radiation detector device, and
 - (c) measuring radiation emission from the radionuclide in the subject, with the radiation detector device, to construct an image of radiation emission, wherein said image is a representation of cell death in said nucleated cell of said mammalian subject.
13. A radiolabeled annexin according to claim 12, wherein the amount of labelled annexin administered is between about 1 and 10 µg annexin protein/kg.
14. A radiolabeled annexin according to claim 12 or 13, wherein said region is in the head of said subject or a portion thereof, or in the heart of said subject or a portion thereof, or in the liver of said subject or a portion thereof.
15. A radiolabeled annexin according to any one of claims 12-14, wherein said region is in a tumour in said subject or a portion thereof, or in a transplant in said subject or a portion thereof, or in an ischemic site in said subject or a portion thereof.

FIG. 1. (SEQ ID No. 7)

10 20 30 40
AQVLRGTVTD FPGFDERADA ETLRKAMKGL GTDEESILTL

50 60 70 80
LTSRSNAQRQ EISAAFKTLF GRDLLDDLKS ELTGKFEKLI

90 100 110 120
VALMKPSRLY DAYELKHALK GAGTNEKVLT EIIASRTPEE

130 140 150 160
LRAIKQVYEE EYGSSLEDDV VGDTSGYYQR MLVVLLQANR

170 180 190 200
DPDAGIDEAQ VEQDAQALFQ AGELKWGTDE EKFITIFGTR

210 220 230 240
SVSHLRKVFD KYMTISGFQI EETIDRETSG NLEQLLLAVV

250 260 270 280
KSIRSPAYL AETLYYAMKG AGTDDHTLIR VMVSRSEIDL

290 300 310 319
FNIRKEFRKN FATSLYSMIK GDTSGDYKKA LLLLCGEDD

FIG. 2 (SEQ ID No. 8)

 16 26 36 46
HHHHHH AQVLRGTVTD FPGFDERADA ETLRKAMKGL GTDEESILTL

 56 66 76 86
LTSRSNAQRQ EISAAFKTLF GRDLLDDLKS ELTGKFEKLI

 96 166 116 126
VALMKPSRLY DAYELKHALK GAGTNEKVL T EIIASRTPEE

 136 146 156 166
LRAIKQVYEE EYGSSLEDDV VGDTS GGYYQR MLVLLQANR

 176 186 196 206
DPDAGIDEAQ VEQDAQALFQ AGELKWGTDE EKFITIFGTR

 216 226 236 246
SVSHLRKVFD KYMTISGFQI EETIDRETSG NLEQLLLAVV

 256 266 276 286
KSIRSIPAYL AETLYYAMKG AGTDDHTLIR VMVSRSEIDL

 296 366 316 325
FNIRKEFRKN FATSLYSMIK GDTSGDYKKA LLLLCGEDD

INTERNATIONAL SEARCH REPORT

International application No

PCT/NL2009/050513

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K51/08 C07K14/47 G01T1/161

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)

A61K C07K G01T

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, WPI Data, EMBL, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>TAIT J F; ET AL: "DEVELOPMENT AND CHARACTERIZATION OF ANNEXIN V MUTANTS WITH ENDOGENEOUS CHELATION SITES FOR 99MTC" BIOCONJUGATE CHEMISTRY, ACS, WASHINGTON, DC, US, vol. 11, no. 6, 1 November 2000 (2000-11-01), pages 918-925, XP009003666 ISSN: 1043-1802 especially introduction p 918, 2nd column lines 19-21 (histidine residues as chelation site) the whole document</p> <p>----- --/--</p>	1-15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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- *E* earlier document but published on or after the international filing date
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- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

5 October 2009

Date of mailing of the international search report

16/10/2009

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 Fax: (+31-70) 340-3016

Authorized officer

Kools, Patrick

INTERNATIONAL SEARCH REPORT

International application No

PCT/NL2009/050513

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>WAIBEL ROBERT; ALBERTO ROGER; WILLUDA JORG; FINNERN RICARDA; SCHIBLI ROGER; STICHELBERGER ALBERT; EGLI ANDRE; ABRAM ULRICH; MACH J: "Stable one-step technetium-99m labeling of His-tagged recombinant proteins with a novel Tc(I)-carbonyl complex"</p> <p>NATURE BIOTECHNOLOGY, vol. 17, no. 9, September 1999 (1999-09), pages 897-901, XP002548771</p> <p>ISSN: 1087-0156</p> <p>abstract</p>	1-15
A	<p>TAIT JONATHAN F ET AL: "Structural requirements for in vivo detection of cell death with Tc-99m-annexin V"</p> <p>JOURNAL OF NUCLEAR MEDICINE, vol. 46, no. 5, May 2005 (2005-05), pages 807-815, XP002510935</p> <p>ISSN: 0161-5505</p> <p>abstract</p>	1-15
A	<p>BERNDORFF DIETMAR ET AL: "Imaging of tumor angiogenesis using Tc-99m-labeled human recombinant Anti-ED-B fibronectin antibody fragments"</p> <p>JOURNAL OF NUCLEAR MEDICINE, vol. 47, no. 10, October 2006 (2006-10), pages 1707-1716, XP002510936</p> <p>ISSN: 0161-5505</p> <p>abstract</p>	1-15
A	<p>CORTEZ-RETAMOZO, ET AL.: "99mTc-labeled nanobodies: A new type of targeted probes for imaging antigen expression"</p> <p>CURRENT RADIOPHARMACEUTICALS, BENTHAM SCIENCE PUBLISHERS, vol. 1, no. 1, January 2008 (2008-01), pages 37-41, XP002510937</p> <p>abstract</p>	1-15
A	<p>BIECHLIN MARIE-LAURE ET AL: "Improvement in radiolabelling proteins with the Tc-99m-tricarbonyl-core [Tc-99m(CO)(3)](+), by thiol-derivatization with iminothiolane: application to gamma-globulins and annexin V"</p> <p>JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS, vol. 48, no. 12, October 2005 (2005-10), pages 873-885, XP002510938</p> <p>ISSN: 0362-4803</p> <p>abstract</p>	1-15
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International application No
PCT/NL2009/050513

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WO 2006/003488 A (I P RANDWIJCK B V [NL]; REUTELINGSPERGER CHRIS [NL]) 12 January 2006 (2006-01-12) cited in the application the whole document -----	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/NL2009/050513

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