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(54) Titre : DERIVES D'ANNEXINE ADAPTES POUR LE PRECIBLAGE EN THERAPIE ET EN DIAGNOSTIC
(54) Title: ANNEXIN DERIVATIVES SUITABLE FOR PRETARGETING IN THERAPY AND DIAGNOSIS

(57) Abrégé/Abstract:
The present invention provides methods and compositions for the treatment and diagnosis of diseases such as neoplastic diseases, neurodegenerative diseases, cardiovascular diseases, autoimmune diseases, and inflammatory diseases. The methods are based on the concept of pretargeting and include the administration of complexes comprising a recognizable compound A coupled to annexins, and the administration of complexes comprising of pharmaceutical or diagnostic compounds coupled to a compound B recognizing and binding to compound A to subjects. The compositions include annexins, annexin variants, that are not internalized by the target cells, derivatives thereof, and complexes thereof.

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Annexin derivatives suitable for pretargeting in therapy and diagnosis

The present invention relates generally to the field of annexins. More particularly, it relates to compositions and methods for treating and diagnosing a subject by delivering compounds to a specified target using novel annexins, variants of annexins, and derivatives thereof that do not form trimers and 2-dimensional networks.

BACKGROUND OF THE INVENTION

Cells are enveloped by a plasma membrane ("PM") that consists of a bilayer of phospholipid molecules and several protein molecules. Various phospholipid molecules form the building blocks of the bilayer. The phospholipid molecules are distributed asymmetrically over the two leaflets of the bilayer. Phosphatidylcholine for example is present in both layers, whereas sphingomyeline can be found only in the outer leaflet facing the environment. Aminophospholipids, like phosphatidylserine ("PS"), on the other hand, are predominantly present in the inner leaflet facing the cell's cytosol (Zwaal and Schroit, *Blood* 89:1121-32 (1997)). Aminophospholipid translocases transport PS from the outer to the inner layer, or leaflet, of the plasma membrane to create an asymmetric distribution of PS. The asymmetric architecture of the PM is a feature of living cells. They expend energy to generate and maintain the uneven distribution of the phospholipid species in their PM's.

A cell can change the phospholipid architecture of its PM under certain circumstances, which lead to activation and perturbation of the cell. Programmed cell death ("PCD") is associated with the appearance of PS in the outer leaflet of the PM (Fadok et al., *J. Immunol.* 148:2207-16 (1992)). On the basis of morphology and biochemistry, at least four types of PCD have been identified: (1) apoptosis, (2) apoptosis-like PCD, (3) necrosis-like PCD, and necrosis. Each type is accompanied by a change in the asymmetry of the PM characterized by exposure of PS to the outer layer of the cell surface. PS exposure at the outer layer of the PM is a good indication of a variety of activated and perturbed states of a cell. PS exposure, however, is not exclusively associated with cellular processes culminating in cell death. Transient and reversible PS exposure has been reported for several cell types, including activated B cells, undifferentiated muscle cells prone to form syncytium, chlamydia infected cells, endothelial cells of tumour vasculature (United States Patent 6,312,694), and engulfing macrophages (Kenis et al. *J. Biol. Chem.* 2004 279: 52623-9). In addition, several cellular processes and conditions have been found that are associated with an expression of PS at the outer leaflet of the PM. These include platelet activation, red blood cell ageing, stimulation of the immune system, muscle cell syncytium formation, new blood vessel formation in tumors (US 6,312,694), and tumor growth (Rao et al., *Thromb. Res.* 67:517-31 (1992)).

In addition, cells can dissipate portions of themselves from their surface resulting in membrane encapsulated microparticles. These microparticles have aminophospholipids such as PS exposed at the outer layer of the membranes. These microparticles have been associated with diseases like infection, AIDS, atherosclerosis. Therefore, aminophospholipids at the cell surface are indicators of a variety of activated and perturbed states of a cell. Moreover, microparticles that exhibit exposed aminophospholipids reflect distant cell activation and perturbation. Hence, phospholipids at the surface of a PM constitute attractive targets for a variety of purposes including research, diagnosis, prevention and treatment of diseases. Preferably, PS in the outer leaflet of a PM constitutes a target for research, diagnosis, prevention and treatment of diseases.

Pharmacological and genetic treatments of diseases are based on the delivery of pharmacologically active compounds to diseased cells where the compounds act preferably intracellularly. Current therapeutic treatments employ systemic delivery of a drug, where the drug circulates through the entire body before reaching its desired target. This method of drug delivery results in systemic dilution of the compound. As a result higher concentrations of the drug are required to achieve a therapeutic efficacy. This is associated with undesired toxic side-effects and increased costs of drugs.

Solutions to these problems are provided by targeted drug delivery systems. The targeting agent, which is coupled to the drugs directly or indirectly, guides the drugs to the diseased cells where they accumulate.

Recently we described annexins, derivatives thereof and annexin-Cys variants as targeting and cell-entry agents and their uses for therapeutic and diagnostic applications (WO 2006/003488, published 12 January 2006). The predominant target of annexins is phosphatidylserine (PS), which is exposed by cells that execute programmed cell death or are submitted to stress such as metabolic stress. The annexins, derivates and annexin-Cys variants as described in WO 2006/003488 bind to cell surface exposed PS and are subsequently internalized. The internalization results in a depletion of surface-bound annexins. This phenomenon disfavours the use of annexins in therapeutic and diagnostic procedures that employ the concept of pretargeting.

Pretargeting is a strategy of targeting a reporting compound for diagnostic purposes and/or a drug for therapeutic purposes to the diseased tissue in a multi-step procedure in order to reduce the background signal and the systemic toxic burden respectively. The pretargeting concept employs two compounds A and B which have a high affinity for each other. Compound A encompasses the targeting function and compound B contains the reporter and/or therapeutic function. Firstly, compound A with the targeting function is administered to the subject. After a certain period of time when the circulating compound A is cleared sufficiently, compound B with the reporter and/or therapeutic drug is given to the subject. The latter compound will accumulate at sites where compound A is retained due to its targeting function. This strategy reduces the amount of compound B that needs to be administered in order to obtain the desired effect. Moreover, it will circumvent background signals and undesired toxic side-effects that

are related to compound A if the reporter and/or therapeutic drug were directly coupled to compound A.

Examples of combinations of A and B compounds that have high affinity for each other and that are suitable for pretargeting include the streptavidin/avidin and biotin combination, combination of complementary DNA and RNA oligonucleotides, complementary DNA and RNA analogs such as morpholinos (synthetic oligonucleotide analogues containing morpholino-phosporodiimidate chains instead of deoxyribose-phosphodiester chains), peptide nucleic acids (synthetic oligonucleotide analogues containing N-aminoethyl-glycine chains instead of deoxyribose-phosphodiester chains, PNA) and aptamers (specifically binding oligonucleotides or oligopeptides), the antibody and hapten combination, and the receptor and ligand combination. These combinations have been used in delivery of radionuclides for imaging and therapy of cancer through the pretargeting strategy (Sharkey et al, *Clin. Cancer Res.* 2005, 11:7109-21).

The prerequisite for successful implementation of the pretargeting strategy is the accessibility of compound A for compound B. Internalization of compound A by the target cell would reduce the efficacy of this strategy.

Patent application WO 2006/003488, describes that annexins, derivatives thereof, and annexin-Cys variants are internalized by cells that expose PS at their surface. The mechanism of internalization is based on the formation of annexin-trimers and the organization of the annexin-trimers in large 2-dimensional networks (Kenis et al. *J. Biol. Chem.* 2004 279: 52623-9). This mechanism, thus, diminishes the efficient use of annexin, derivatives thereof, and annexin-Cys variants as targeting parts of compound A in pretargeting strategies.

Mira et al., *J. Biol. Chem.* 1997, 272: 10474-82, describe annexin mutants M1-M4, which affect Ca^{2+} binding and the effect thereof on inhibition of cytosolic phospholipase A_2.

SUMMARY OF THE INVENTION

In accordance with one embodiment of the invention there is provided annexin variants that are suitable for pretargeting strategies for diagnosing and treating diseases. These annexin variants bind PS with sufficient affinity and are unable to form trimers and 2-dimensional networks on the cellular surface and, hence, they do not induce their own internalization.

Another embodiment of the invention concerns annexins and variants thereof that are derivatised with affinity compounds A for pretargeting strategies. Such affinity compounds include biotin, compounds containing one or more biotin groups, streptavidin, avidin, DNA oligonucleotides, RNA oligonucleotides, morpholinos, PNA's, aptamers, receptors, compounds with high affinity for receptors and immuno-

globulins or parts thereof. In the present invention, affinity compound A is also referred to as recognizable compound (A), or as compound A, with the same meaning.

A further embodiment of the invention comprises annexins, derivatives thereof and annexin-Cys variants according to patent application WO 2006/003488 that are derivatised with affinity compounds A via conjugation to the cysteine residue.

One embodiment of the invention relates to the use of affinity compounds B that are conjugated with fluorescent compounds, radionuclides, MRI contrast agents, CT contrast agents, cytostatics, and therapeutic biologicals including cytokines, complement factors, toxins, and immunoglobulins in combination with the annexin derivatives and complexes. Affinity compounds B have a high affinity for affinity compounds A of other embodiments of the invention. In the present invention, affinity compound B is also referred to as compound (B) recognizing compound A, or as recognizing compound B, or compound B, with the same meaning.

One embodiment of the present invention is a kit that includes at least one complex of annexin derivative with affinity compound A described above and, optionally at least one pharmaceutically acceptable excipient.

Another embodiment of the invention relates to a method for delivering a pharmaceutical compound to a target cell that includes administering firstly a targeting complex of the composition that is described above and secondly a therapeutic complex of the composition that is described above. More specifically, this embodiment encompasses a method to treat or prevent a disease, where the pharmaceutical compound is a therapeutic compound that is effective to treat or prevent the disease.

One embodiment of the invention relates to a method for delivering a diagnostic compound to a target cell that includes administering firstly a targeting complex of the composition that is described above and secondly a diagnostic complex of the composition that is described above. More specifically this embodiment encompasses a method to diagnose a disease and to determine the efficacy of a therapeutic treatment, where the diagnostic compound is a molecular imaging compound that can be detected by imaging modalities comprising optical imaging, nuclear imaging, MRI, CT and ultrasound.

For a better understanding of the present invention, together with other and further objects thereof, reference is made to the following description, taken in conjunction with the accompanying drawings, and its scope will be pointed out in the appending claims.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based in part on the directed search to identify those amino acids that are involved in the inter-molecular interactions between annexin molecules. Molecular modelling and docking of the crystal structures available in the Protein Database (PDB, 1AVR and ANX) revealed amino acids that are involved in the trimer

formation. These amino acids are found in the helices IA, ID, IIA, IID, IIIC, IIID and IVE and in the stretches connecting helices IC and ID, IIE and IIIA, IIIC and IIID, IIID and IIIE, and IVA and IVB (for localization of these helices in the annexin A5 molecule see Huber et al. *EMBO J.* 12:3867-74 (1990)). The annexin variants of one of the embodiments of the invention have one or more amino acids replaced in the helices IA, ID, IIA, IID, IIIC, IIID and IVE and in the stretches connecting helices IC and ID, IIE and IIIA, IIIC and IIID, IIID and IIIE, and IVA and IVB so as to impair their ability to form trimers and a 2-dimensional network on the cellular surface and consequently to impair their internalization into the cell. The annexin variants will remain longer on the cellular surface and are, thus, suitable for pretargeting strategies.

Thus, the invention generally pertains to an annexin variant, a) that binds to at least one phospholipid, in particular to phosphatidylserine (PS), and b) that is not internalized into a cell. The term “annexin” refers to any protein capable of binding to phospholipids, especially phosphatidylserine, and member of the so-called annexin family. The family covers many members; information thereon and on the protein and nucleotide sequences can for example be found on http://snoops.bch.ed.ac.uk/annexins/seq/-search.php. By way of example, reference is made herein to annexin A5, having the amino acid sequence of SEQ ID No. 1, but other annexins can equally be used for producing and using the annexin variants of the invention. Figure 2 contains an alignment of human annexins A1 to A11 and A13. Some of the annexins, such as A1, A6, A7 and A11 have long extensions at the N-terminus. These parts have not been included in Figure 2, and are believed to be less relevant for the purpose of the invention. Here below, reference is made to the amino acid sequence and the positions of annexin A5, but what applies to A5 also applies to the other annexins, especially human annexins, by choosing the corresponding position found with the alignment of Figure 2 or corresponding alignment of any annexins not represented in Figure 2.

Specific annexin variants according to the invention have amino acid sequences according to Figure 1 (SEQ ID NO:1), which are modified to inhibit the internalization into a cell in that one or more amino acids within the helices and connecting stretches indicated above are replaced by different amino acids. These amino acids are located at positions 16-29, 59-74, 88-102, 135-145, 156-169, 202-231, 259-266, and 305-317 in annexin A5, and these positions are underlined in SEQ ID No. 1 and in Figure 2. Where the annexin variant contains one or more of these modifications, the feature of the annexin not being internalized into a cell is considered as being fulfilled. Thus, the invention also comprises an annexin variant that a) binds to at least one phospholipids and b) contains one or more of the amino acid modifications as described herein.

Preferred modifications are substitutions, especially substitutions of polar amino acids by non-polar amino acids. Thus, preferred amino acids for substitution include arginine (R), lysine (K), aspartate (D), glutamate (E), asparagine (N) and glutamine (Q). They may be substituted e.g. by alanine (A) or glycine (G), or by a non-polar amino acid that is located in the corresponding position of another annexin (cf. Figure 2). Suitable

examples of substituted amino acids include E21, K25 (e.g. by G, T) , R62 (e.g. G, A), D63 (e.g. G, A, P), K69, D91, K96, H97, K100, E137 (e.g. A, G, V), D138, D139, N159 (e.g. A, G, S), R160, R206, K207, Q219, D225, R226, D264, K308, K309. The modifications M1-M4, at positions E71, D143, E227 and D302, are less preferred according to the invention.

It is preferred to have at least two, or even at least three, substitutions in different regions, for example R62A + E137G, or K69A + K100A + N159S etc., in order to further decrease the trimerisation of the annexin at the site of the cell.

The annexin variants according may further comprise one or more amino acid substitutions, deletions, or additions, wherein the amino acid substitutions, deletions, or additions do not substantially affect the ability of the annexin variant to bind to at least one phospholipid, and to bind at least on recognizable compound A, and wherein the amino acid substitutions, deletions, or additions do not substantially affect the inhibited internalization of the recognizable compound A into a cell.

The substitution must be such that the annexin still binds sufficiently to the phospholipid. Sufficient binding means a dissociation constant of approximately 10^{-6} M or less in the presence of Ca^{2+}-ions.

The invention further pertains to annexin molecules or variants as defined above to which a recognizable compound A, wherein the annexin variant is not internalized by the target cell. The recognizable compound A may for example be a biotin or a complex of multiple biotins. Other suitable examples include avidin or streptavidin, an oligonucleotide or a nuclease-resistant oligonucleotide analogue in the form of a morpholino compound or a PNA or an aptamer.

In another embodiment the recognizable compound A is a receptor or a part thereof, or a receptor ligand or a part thereof. Other examples of recognizable compounds A include an antibody or a fragment thereof, for example a nanobody – a truncated antibody from camel-like origin - , or an antigen.

The recognizable compound can be bound to the annexin by methods known per se. One method consists in covalently binding the recognizable compound to specific amino acids, possibly in derivatised form. Especially suitable is binding to a cysteine residue of the annexin, for example in the case of biotin, which can be derivatised with a maleimide group. In order not to interfere with functional properties of the annexin, the amino acid to which the recognizable compound is coupled is located at the concave sides of the annexin molecule. These are represented by *italic* amino acid symbols in SEQ ID NO. 1. Moreover, these positions may or may not be located in the regions selected above for preventing trimerisation and internalization of the annexin. Thus the preferred sites are the bold italic positions in SEQ ID No. 1, i.e. the stretches 1-15, 46-58, 86-87, 118-134, (170), 245-248 and 280-294 of annexin A5 and the corresponding stretches in other annexins (see Figure 2), but underlined italic positions

(16-19, 24, 28, 59-64, 88-89, 135, 157-169, 203-219) can also be used for introducing cysteine residues.

It is furthermore preferred that any cysteine residues naturally present outside these stretches, especially those which are present in the parts where amino acids are substituted according to the invention for the purpose of preventing trimerisation, are replaced by other amino acids. Such other amino acid can be a neutral small amino acid such as G, A or S, or an amino acid that is present at the same position ion of another annexin. For example, C107 of annexin A4 can suitably be replaced by V or A, C201 of annexin A3 (and the counterparts in many other annexins) can be replaced by G or A, and C315 in annexin A5 (and the counterparts in many other annexins) can be replaced by V, A or S. The C292 of annexin A8 need not be replaced as it is in a position suitable for derivatisation. The amino acid substitutions can be performed by recombinant techniques well-known in the art and illustrated in the examples below.

Thus the invention pertains to an annexin variant, which contains a cysteine residue at one of the amino acid positions 1-19, 24, 28, 46-64, 86-89, 118-135, 149-150, 157-170, 203-219, 245-248 and 280-294, and does not contain a cysteine residue outside these positions, and which furthermore contains substitution of one or more amino acids Lys, Arg, Gln, Asn, Glu, Asp or His at positions 16-29, 59-74, 88-102, 135-145, 156-169, 202-231, 259-266 and 305-317 by Gly, Ala, Val, Ile, Leu, Ser, Thr, Met, Pro, Phe, or Tyr, preferably Gly, Ala, Val or Ser; herein the corresponding amino acid positions in other annexins apply, e.g. as depicted in Figure 2. The annexins may be the proteins as such, or the conjugates with spacers and/or recognizable compounds. The cysteine residue(s) may be substituted, e.g. with a recognizable group (A).

The complexes of the annexin variant and the recognizable compound A can be used in a therapeutic or diagnostic method for targeting a medicament or a diagnostic agent to a specific site, in particular to cells which expose PS. In such a method, a composition containing the complex of the annexin variant and the recognizable compound A is first administered to a subject for which such therapeutic or diagnostic method is intended, followed by administering to the subject a composition comprising at least one complex comprising a compound B recognizing and binding to compound A and a diagnostic or therapeutic compound. Compound B is especially a specific counterpart of compound A, for example streptavidin or avidin, in case compound A is biotin. Likewise, compound B may be biotin or a complex of multiple biotins, especially if compound A is streptavidin or avidin. Compound B may also be an oligonucleotide, a morpholino, a PNA or an aptamer which have high affinity for the oligonucleotide, morpholino, PNA or aptamer counterpart bound to the annexin molecule as described above. It may also be a receptor or a part thereof, where compound A is the receptor ligand, or vice versa. Also compound may be an antigen to an antibody as compound A or fragment thereof or vice versa.

The diagnostic agent that can be used in the diagnostic method of the invention, can be selected from a fluorescent group, a radionuclide, an MRI contrast agent, a CT contrast

agent, an ultrasound agent, and a combination thereof. Suitable examples of fluorescent groups are fluoresceines, Alexas, Phycoerythrines, Cy-compounds, Nanocrystals and a combination thereof. Suitable examples of radionuclides include Carbon-11, Fluorine-18, Indium-111, Iodine-123, Iodine-131, Nitrogen-13, Oxygen-15, Technetium-99m, Zirconium-89, Ga-67, Ga-68, Cu-64 and a combination thereof, which are incorporated in suitable molecules bound to compound B or in compound B itself. An MRI contrast agent may be selected from Gadolinium, magnetic particles and paramagnetic particles.

Therapeutic compounds that can be used in the therapeutic method of the invention, can for example be a toxin, an enzyme, enzyme inhibitors, 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, and a combination thereof. The toxin can be selected from Dt, PE, P38, P40, ricin, abrin, diphtheria toxin, cholera toxin, gelonin, pseudomonas, exotoxin, shigella toxin, pokeweed antiviral protein, and a combination thereof. Examples of enzymes that can be coupled to compound B include peroxidases, alkalases, caspases, and a combination thereof. Lipids can e.g. be selected from phospholipids, fatty acids, terpenes, steroids, and a combination thereof. The lipid can be embedded in the membrane of a liposome.

Examples of chemotherapeutic agents include BiCNU, bleomycin, busulfan, CCNU, carboplatin, carmustine, cisplatin, cisplatinum, chlorambucil, 2-cholrodeoxyadenosine, cladirabine, cytarabine, cyclophosphamide, dacarbazine, daunorubicin, docetaxel, doxorubicin, DTIC, etoposide, 5-flourouracil, fludarabine, gemcitabine, hydroxyurea, idarubicin, ifosfamide, irinotecan, lomustine, melphelan, methotrexate, mithramycin, mitomycin, mitoxantrone, nitrogen mustard, oxaliplatin, paclitaxel, plicamycin, procarbazine, raltritexed, semustine, tomudex, topotecan, vinblastine, vincristine, vinorelbine, and combinations thereof.

Examples of photosensitizers include phthalocyanines, rhodoporphyrins, rhodochlorins, mesorhodochlorins, phylloerythrin and its derivatives, porphorin and its derivatives, metal-pyrollic compounds, and combinations thereof.

Cell death inducing agents that can advantageously be used in the method of the invention can be selected from the group of apoptosis inducers, kinase inhibitors, activators of mitochondrial permeability transition activators, polynucleotides encoding for a cell death inducing protein, activators of ion-transport across the membrane, polynucleotides being an anti-sense to polynucleotides encoding for cell death inhibiting proteins, polynucleotides interacting with and inhibiting cell death inhibiting proteins, and a combination thereof.

Examples of therapeutic radionuclides include ^{32}P, ^{89}Sr, ^{90}Y, ^{103}Pd, ^{125}I, ^{131}I, ^{137}Cs, ^{153}Sm, ^{186}Re, ^{188}Re, ^{192}Ir, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{86}Y, ^{105}Rh, ^{111}In, ^{114m}In, ^{124}I, ^{149}Pm, ^{166}Ho, ^{169}Yb, ^{177}Lu, ^{211}At, ^{213}Bi, ^{225}A.

The therapeutic and diagnostic compounds can be bound to compound B by methods known in the art. For example, when the therapeutic or diagnostic compound is a protein, it can be coupled through a lysine or arginine residue, optionally after activation using cyanogen bromide or other chemical or physical methods. If the compound is a lipid, such as phosphatidylethanolamine, it can be coupled to the amino group using methods known in the art. If the compound is a polynucleotide it can be coupled for example to hydroxyl groups thereof using methods known in the art. If the compound is a radionuclide, it can be coupled either directly or indirectly by coupling a chelator to compound B that chelates the radionuclide of choice.

The complex can also be used for detecting the presence or absence of cells or cell particles expressing phospholipids comprising:
a) administering to a subject a composition comprising at least one complex comprising a recognizable compound A and an annexin or an annexin variant, and :
b) administering to a subject a composition comprising at least one complex comprising a compound B recognizing compound A and a diagnostic agent, and:
c) submitting a subject to a detecting step such as optical imaging, SPECT imaging, PET imaging, MRI imaging, CT imaging, and ultrasound imaging.

The invention also pertains to a diagnostic kit suitable for carrying out the diagnostic method as described above, which comprises at least a complex of an annexin variant and an affinity compound A, and optionally a complex of a detectable (reporter) compound and an affinity compound B, and optionally diluents and further components necessary for carrying a diagnostic method. Preferably, the complex of the annexin variant and the complex of the detectable compound are conditioned separately.

The invention also pertains to a pharmaceutical kit suitable for carrying out the therapeutic method as described above, which comprises at least a complex of an annexin variant and an affinity compound A combined with a pharmaceutically acceptable excipient, and optionally a complex of a therapeutic compound and an affinity compound B, and optionally diluents and further components necessary for carrying a therapeutic method. Preferably, the complex of the annexin variant and the complex of the therapeutic compound are conditioned separately.

The present invention provides methods and compositions for the treatment, diagnosis, prevention, and research of diseases, such as neoplastic diseases, neurodegenerative diseases, cardiovascular diseases, autoimmune diseases, and inflammatory diseases. The methods include the administration to subjects of targeting complexes comprising annexins and annexin variants and diagnostic and therapeutic complexes comprising molecular imaging agents and pharmaceutical compounds respectively.

The present invention relates to the ability of annexins to bind to PS expressing cells. The present invention relates to the use of annexins in pretargeting methods to diagnose and treat diseases. It is known that annexins are taken up by liver, spleen and kidneys and by the reticulo-endothelial system of the bone marrow. Injecting annexins

conjugated to diagnostic compounds and therapeutic compounds will result in high background signals and undesired toxic side-effects respectively. Therefore, the present invention provides methods in which annexins will be conjugated neither to diagnostic compounds such as fluorescent groups, radionuclides, MRI contrast agents, CT contrast agents and ultrasound agents nor to therapeutic compounds such as 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 prior to their administration into the subject. Instead, the present invention provides methods in which annexins be coupled to recognizable compounds A, which are selected from the set comprising but not restricted to biotin, multiple biotins, streptavidin, avidin, DNA, RNA, morpholinos, PNAs, aptamers, receptors and receptor ligands and antibodies. The present invention provides methods for delivering a diagnostic or a therapeutic compound to a target cell by delivering a complex of an annexin and a recognizable compound A to the target cell and by delivering subsequently a complex of a compound B that recognizes compound A and a diagnostic compound or a therapeutic compound to the target cell. The conjugated annexins will be administered to the subject. After a period of time, for example between 1 h and 24 h, the diagnostic or the therapeutic complex with compound B, which has a high affinity for compound A, will be injected into the same subject. Compound B will accumulate at sites where annexins conjugated to compound A are bound to cellular surfaces.

Annexins constitute a multigene family of proteins that share structural and functional features. The annexin polypeptide is organized in domains that form the so-called Annexin fold in space (Gerke and Moss, *Physiol. Rev.* 82:331-71 (2002)). The domains contain calcium binding sites through which an interaction with phospholipid membranes can occur. Once bound to a phospholipid surface the annexins can form a two-dimensional lattice through protein-protein interactions (Oling et al., *J. Mol. Biol.* 304: 561-73 (2001)). The physiological significances of the annexins are poorly understood but are thought to be related to their phospholipid binding activity. The annexins do not have a signal sequence and are therefore thought to play a role within the cell. Extracellular localization of annexins has been reported but it is unknown whether this has happened by a selective process or by an aspecific event such as cell lysis. According to WO 2006/003488, annexins and annexin-Cys variants will induce their own internalization after binding to cell surface expressed PS. The phenomenon of internalization reduces the efficacy of the pretargeting strategy. The present invention relates to annexins that are not internalized by the target cells. In order to find annexin variants that are less internalized the mechanism of internalization was inspected on its structure-function relationships. The internalization is induced by the formation of annexin trimers that form a 2-dimensional network on the phospholipid bilayer (Kenis et al., *J. Biol. Chem.* 279:52623-9 (2004)). The annexin trimers arise from non-covalent interactions between annexin molecules.

Example 1: Production of Annexin A5-2D variants having one or more amino acids at positions 62, 69, 100, 137, 138 and 159 replaced by Alanine and Glycine.

The human annexin A5 cDNA was prepared from a white blood cell cDNA library from a healthy volunteer with standard techniques known in the art. The cDNA sequence
5 encoded the amino acid sequence presented in Figure 1. Primers were designed to mutate annexin A5 by PCR techniques such that the resulting cDNA encoded the amino acid sequence of Figure 1 with the exception of the following substitutions, that were performed singularly, but also in combinations thereof in the cDNA: R62A, K69A, K100A, E137A, D138G, and N159A. The annotation employs the single letter code for
10 amino acids and the numerical position in the amino acid sequence where the substitution occurs with left to the number presenting the original amino acid and right to the number presenting the substitute.
The annexin A5-2D cDNA was cloned into a bacterial expression vector with standard techniques known in the art. *E. coli* transformed with the resulting bacterial expression
15 vectors were grown in a fermentor. The annexin A5-2D variants that were produced by the bacteria were isolated and purified from *E. coli* lysates with standard chromatography techniques known to persons skilled in the art.
The purified annexin A5-2D variant appeared as a homogenous band of around 34kDa on SDS-PAGE and exhibited full calcium-dependent phosphatidylserine binding
20 activity as measured by plasmon surface resonance technique using the BiaCore.

Example 2: Production of Annexin A5-2D variants having one or more amino acids at positions 62, 69, 100, 137, 138 and 159 replaced by Alanine and Glycine and having Glutamine at position 2 replaced by Cysteine.

The annexin A5-2D cDNA was prepared as described in Example 1 of the present
25 invention. Primers were designed to mutate annexin A5-2D cDNA by PCR techniques such that the resulting cDNA encoded the amino acid sequence of an annexin A5-2D variant with the exception that the amino acid Glutamine at position 2 was replaced by the amino acid Cysteine.
The purified annexin A5-2D-Cys2 variant appeared as a homogenous band of around
30 34kDa on SDS-PAGE and exhibited full calcium-dependent phosphatidylserine binding activity as measured by plasmon surface resonance technique using the BiaCore.

Example 3: Production of Annexin A5-2D variants having one or more amino acids at positions 62, 69, 100, 137, 138 and 159 replaced by Alanine and Glycine and having Glycine at position 165 replaced by Cysteine.

35 The annexin A5-2D cDNA was prepared as described in Example 1 of the present invention. Primers were designed to mutate annexin A5-2D cDNA by PCR techniques such that the resulting cDNA encoded the amino acid sequence of an annexin A5-2D variant with the exception that the amino acid Glycine at position 165 was replaced by the amino acid Cysteine.

The purified annexin A5-2D-Cys165 variant appeared as a homogenous band of around 34kDa on SDS-PAGE and exhibited full calcium-dependent phosphatidylserine binding activity as measured by plasmon surface resonance technique using the BiaCore.

Example 4: Binding of Annexin A5-2D variants having one or more amino acids at positions 62, 69, 100, 137, 138 and 159 replaced by Alanine and Glycine to phospholipid bilayers.

This example demonstrates that the annexin A5-2D variant has a calcium-dependent ability to bind to PS of phospholipid bilayers like annexin A5 but lacks the ability to form a 2-dimensional network on the phospholipid surface and is not internalized into a cell.

The binding of annexin A5-2D to a phospholipid bilayer containing PS was studied by ellipsometry (Andree et al., *J. Biol. Chem.* 265:4923-4928 (1990)). In the absence of calcium the annexin A5-2D variant did not bind to the phospholipid surface by increasing the calcium concentration an increase in binding was observed similar to the calcium-dependent binding isotherm of annexin A5.

Annexin A5-2D bound to a phospholipid surface was analysed by electron microscopy (Mosser et al., *J. Mol. Biol.* 271:241-5 (1991)). Unlike annexin A5, the annexin A5-2D variant did not form an ordered 2-dimensional network.

Jurkat cells were co-incubated with fluorescent annexin A5-2D or fluorescent annexin A5, and the apoptotic stimulus. The cells were analysed for the localization of fluorescent annexin A5-2D or fluorescent annexin A5 by confocal scanning laser microscopy (Kenis et al., *J. Biol. Chem.* 279:52623-9 (2004)). Annexin A5 was internalized. Annexin A5-2D was not internalized but remained bound to the plasma membrane.

Example 5: The coupling of maleimide-activated biotin to the Annexin A5-2D variant having amino acids at positions 62, 69, 100, 137, 138 and 159 replaced by Alanine and Glycine and having Glutamine at position 2 and Cysteine at position 315 replaced by Cysteine and Serine respectively.

The annexin A5-2D variant was prepared as described by example 2. The Cysteine was removed at position 315 and incorporated at position 2 in order to be able to couple compounds easily to the annexin A5-2D variant through thiol-chemistry without affecting the PS binding activity of the annnexin A5-2D variant.

EZ-Link PEO-Maleimide activated biotin (Pierce) was dissolved in 25 mM Hepes/-NaOH, pH 7.0, 140 mM NaCl, 1 mM EDTA at a concentration of 10 mM. 3.4 mg/ml annexin A5-2D variant was dialysed into 25 mM Hepes/NaOH, pH 7.0, 140 mM NaCl, 1 mM EDTA. 200 µl biotin solution was added to 1 ml annexin A5-2D variant. The mixture was incubated for 120 minutes at 37°C and thereafter dialysed into 25 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 1 mM EDTA.

The resulting conjugate was assayed on its PS binding activity by ellipsometry and tested for the accessibility of the biotin group using avidin (Pierce). The biotinylated

annexin A5-2D showed no impaired PS binding, while the avidin readily bound to the biotinylated annexin A5-2D on the phospholipid surface.

Example 6: The coupling of maleimide-activated avidin to the Annexin A5-2D variant having amino acids at positions 62, 69, 100, 137, 138 and 159 replaced by Alanine and Glycine and having Glutamine at position 2 and Cysteine at position 315 replaced by Cysteine and Serine respectively.

The annexin A5-2D variant was prepared as described by example 2. The Cysteine was removed at position 315 and incorporated at position 2 in order to be able to couple compounds easily to the annexin A5-2D variant through thiol chemistry without affecting the PS binding activity of the annnexin A5-2D variant.
Immunopure avidin (Pierce) was dissolved in 25 mM Hepes/NaOH, pH 7.4, 140 mM NaCl at a concentration of 8 mg/ml. 1 mg of Sulfo-SMCC (Pierce) was added to the avidin solution and the mixture was incubated for 60 minutes at room temperature. The excess cross-linker was removed by gel-filtration on a PD10 column (GE-Amersham/-Pharmacia). The maleimide activated avidin was added to 1 mg/ml of annexin A5-2D variant that was dialysed into 25 mM Hepes/NaOH, pH 7.0, 140 mM NaCl, 1 mM EDTA. The mixture was incubated for 120 minutes at 37°C.
The conjugate between avidin and the annexin A5-2D variant was tested on its ability to bind to PS by ellipsometry. The conjugate showed no imapired PS binding properties.

Example 7: A procedure to visualize a tumor in vivo using annexin A5-2D variant and pretargeting.

Annexin A5-2D variants having a Cysteine at position 2 or 165 were engineered and produced as presented in examples 2 and 3, respectively. The Cys-annexin A5-2D variants were biotinylated using maleimide-biotin as described by example 5.
Biotinylated annexin A5-2D was injected intravenously into a tumor-bearing mouse. Levels of circulating biotinylated annexin A5-2D were decreased either by time-lapsed spontaneous clearance or by forced clearance with for example intravenously administered avidin. Streptavidin conjugated to a molecular imaging probe such as for example a fluorescent compound or a radionuclide was injected intravenously. The mouse was then subjected to imaging using a whole body optical imager if streptavidin conjugated to a fluorescent probe was used or using a SPECT, PET, SPECT/CT or PET/CT imager if streptavidin conjugated to a radionuclide was used.
This visualization procedure can be applied for localizing and quantifying tumors and metastases and for determination of the efficacy of an anti-tumor therapy.

Example 8: A procedure to visualize unstable atherosclerotic plaques in vivo using annexin A5-2D variant and pretargeting.

Annexin A5-2D variants having a Cysteine at position 2 or 165 were engineered and produced as presented in examples 2 and 3, respectively. The Cys-annexin A5-2D variants were biotinylated using maleimide-biotin as described by example 5.

Biotinylated annexin A5-2D was injected intravenously into a mouse suffering from atherosclerotic lesions. Levels of circulating biotinylated annexin A5-2D were decreased either by time-lapsed spontaneous clearance or by forced clearance with for example intravenously administered avidin. Streptavidin conjugated to a molecular imaging probe such as for example a fluorescent compound or a radionuclide was injected intravenously. The mouse was then subjected to imaging using a whole body optical imager if streptavidin conjugated to a fluorescent probe was used or using a SPECT, PET, SPECT/CT or PET/CT imager if streptavidin conjugated to a radionuclide was used.

This visualization procedure can be applied for localizing unstable atherosclerotic plaques and distinguishing unstable atherosclerotic plaques from stable atherosclerotic plaques. The visualization procedure can be applied to determine the efficacy of drugs that stabilize unstable atherosclerotic plaques.

Example 9: A procedure to treat a tumor in vivo using annexin A5-2D variant and pretargeting.

Annexin A5-2D variants having a Cysteine at position 2 or 165 were engineered and produced as presented in examples 2 and 3, respectively. The Cys-annexin A5-2D variants were biotinylated using maleimide-biotin as described by example 5.

Biotinylated annexin A5-2D was injected intravenously into a tumor-bearing mouse. Levels of circulating biotinylated annexin A5-2D were decreased either by time-lapsed spontaneous clearance or by forced clearance with for example intravenously administered avidin. Streptavidin conjugated to an antic-cancer compound such as for example doxorubicin and cisplatin, or conjugated to a carrier of anti-cancer compounds such as for example liposomes encapsulating for example doxorubicin and cisplatin was injected intravenously.

This pretargeting therapeutic procedure can be applied to delivering locally anti-cancer drugs to the tumor.

Example 10: A procedure to treat unstable atherosclerotic plaques in vivo using annexin A5-2D variant and pretargeting.

Annexin A5-2D variants having a Cysteine at position 2 or 165 were engineered and produced as presented in examples 2 and 3, respectively. The Cys-annexin A5-2D variants were biotinylated using maleimide-biotin as described by example 5.

Biotinylated annexin A5-2D was injected intravenously into a mouse suffering from atherosclerotic lesions. Levels of circulating biotinylated annexin A5-2D were decreased either by time-lapsed spontaneous clearance or by forced clearance with for example intravenously administered avidin. Streptavidin conjugated to atherosclerotic plaque stabilizing compound such as for example statins and anti-inflammatory compounds, or conjugated to a carrier of atherosclerotic plaque stabilizing compounds such as for example liposomes encapsulating for example statins and anti-inflammatory compounds was injected intravenously.

This pretargeting therapeutic procedure can be applied to delivering locally atherosclerotic plaque stabilizing compounds to the atherosclerotic plaques.

All of the examples, methods and/or compositions disclosed, and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the methods and compositions of this invention have been described in terms of preferred embodiments, it will be apparent to those skilled in the art that variations may be applied to the methods and/or compositions and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. It will be apparent to those skilled in the art that compositions with compounds which are structurally and functionally related may be substituted for compositions with the compounds described herein.

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Claims:

1. An annexin variant:
a) that binds to at least one phospholipid;
b) wherein one or more amino acids in the helices IA, ID, IIA, IID, IIIC, IIID and IVE and in the stretches connecting helices IC and ID, IIE and IIIA, IIIC and IIID, IIID and IIIE, and IVA and IVB are replaced by other amino acids, so as to impair internalisation of the annexin variant into a cell,
c) that is bound to a recognizable compound.

2. The annexin variant according to claim 1, wherein the annexin comprises SEQ ID NO:1 for annexin A5 or the corresponding sequences for other annexins, wherein one or amino acids are replaced by other amino acids to inhibit the internalization into a cell.

3. The annexin variant according to claim 2, wherein the one or more amino acids are located at positions 16-29, 59-74, 88-102, 135-145, 156-169, 202-231, 259-266 and 305-317, or the corresponding sequences for other annexins.

4. The annexin variant according to claim 2 or 3, wherein the one or more amino acids are polar amino acids Glu, Gln, Asp, Asn, Arg and/or Lys, and are replaced by a non-polar amino acid.

5. The annexin variant according to any one of claims 1-4, wherein the affinity compound is selected from one or more biotins, avidin or streptavidin, oligonucleotides or morpholinos, peptide nucleic acids and aptamers, receptors or parts thereof, receptor ligands or parts thereof, antibodies or fragments thereof, and antigens.

6. The annexin variant according to any one of claims 1-5, wherein the annexin is bound through a cysteine residue at one of the amino acid positions 1-15, 46-58, 86-87, 118-134, 162-167, 245-248 and 280-294 of annexin A5, and the annexin does not have a cysteine residue at the positions 20-23, 25-27, 29-45, 65-85, 90-117, 136-148, 151-156, 171-202, 220-244, 249-279 and 295-319 of annexin A5.

7. An annexin variant wherein the annexin comprises SEQ ID NO:1 for annexin A5 or the corresponding sequences for other annexins, wherein one or more amino acids at positions 16-29, 59-74, 88-102, 135-145, 156-169, 202-231, 259-266 and 305-317, or the corresponding sequences for other annexins, are replaced by other amino acids, and wherein one or amino acids at positions 1-19, 24, 28, 46-64, 86-89, 118-135, 149-150, 157-170, 203-219, 245-248 and 280-294 has been replaced by a cysteine residue, which is optionally substituted.

8. A method for delivering a diagnostic compound to a target cell in a subject comprising:
a) administering to the subject a composition comprising at least one annexin variant according to any one of claims 1-6,
and
b) administering to the subject a composition comprising at least one complex of a compound (B) recognizing and binding to the recognizable compound (A) bound to the annexin variant, and a diagnostic compound.

9. The method according to claim 8, wherein the diagnostic agent is selected from the group consisting of a fluorescent group, a radionuclide, an MRI contrast agent, a CT contrast agent, an ultrasound agent, and a combination thereof.

10. A method for delivering a pharmaceutical compound to a target cell in a subject comprising:
a) administering to the subject a composition comprising at least one annexin variant according to any one of claims 1-6,
and
b) administering to the subject a composition comprising at least one complex of a compound (B) recognizing and binding to the recognizable compound (A) bound to the annexin variant, and a pharmaceutical compound.

11. The method according to claim 10, wherein the pharmaceutical compound is selected from the group consisting of a toxin, an enzyme, an enzyme inhibitor, 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, and a combination thereof.

12. The method according to any one of claims 8-11, wherein the recognizing compound (B) is selected from streptavidin or avidin, biotin or a complex of multiple biotins, an oligonucleotide or a morpholino, peptide nucleic acids and aptamers, a receptor or a part thereof, a receptor ligand or a part thereof, an antibody or a fragment thereof, and an antigen.

13. A method for detecting the presence or absence of cells or cell particles expressing phospholipids comprising:
a) administering to a subject a composition comprising at least one complex comprising a recognizable compound A and an annexin or an annexin variant, and
b) administering to a subject a composition comprising at least one complex comprising a compound B recognizing compound A and a diagnostic agent, and
c) submitting a subject to a detecting step selected from the group consisting of optical imaging, SPECT imaging, PET imaging, MRI imaging, CT imaging, and ultrasound imaging.

14. A kit comprising:
a) at least one annexin variant according to any one of claims 1-7; and,
b) a diagnostic or therapeutic compound capable of recognizing the annexin variant.

15. Use of an annexin variant according to any one of claims 1-7 in diagnosis.

16. Use of an annexin variant according to any one of claims 1-7 in therapy.