Phosphatidylserine targeting for diagnosis and treatment of human diseases

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Abstract Cells are able to execute apoptosis by activating series of specific biochemical reactions. One of the most prominent characteristics of cell death is the externalization of phosphatidylserine (PS), which in healthy cells resides predominantly in the inner leaflet of the plasma membrane. These features have made PS-externalization a well-explored phenomenon to image cell death for diagnostic purposes. In addition, it was demonstrated that under certain conditions viable cells express PS at their surface such as endothelial cells of tumor blood vessels, stressed tumor cells and hypoxic cardiomyocytes. Hence, PS has become a potential target for therapeutic strategies aiming at Targeted Drug Delivery. In this review we highlight the biomarker PS and various PS-binding compounds that have been employed to target PS for diagnostic purposes. We emphasize the 35 kD human protein annexin A5, that has been developed as a Molecular Imaging agent to measure cell death in vitro, and non-invasively in vivo in animal models and in patients with cardiovascular diseases and cancer. Recently focus has shifted from diagnostic towards therapeutic applications employing annexin A5 in strategies to deliver drugs to cells that express PS at their surface.

Keywords Apoptosis · Phosphatidylserine · Annexin A5 · Molecular Imaging · Targeted Drug Delivery

Introduction

Scientific and technological developments of the past decade have been directed towards the unraveling of molecular fingerprints of distinct diseases in order to facilitate diagnosis and pharmacotherapy by Molecular Imaging (MI) and Targeted Drug Delivery (TDD), respectively. Phosphatidylserine (PS) is one of the molecules that has gained special attention as being part of a cell surface fingerprint of stressed and dying cells. PS is a negatively charged amphotropic lipid that is present in all cells and constitutes approximately 2–10% of total cellular lipid [1]. In addition to a structural function PS is involved in signaling pathways such as protein kinase C pathways [2] and in localizing intracellular proteins to cytosolic membrane leaflets [3]. PS is normally localized in membrane leaflets that face the cytosol. However, certain conditions can cause translocation of PS to the outer leaflet of the plasma membrane where it may initiate and participate in humoral and cellular processes such as blood coagulation and phagocytosis. Cell surface expression of PS has been found with aging erythrocytes, activated platelets, activated macrophages, endothelial cells of tumor blood vessels, apoptotic cells, apoptotic bodies and cell derived microparticles. It is generally believed that phagocytes in healthy tissues rapidly and efficiently remove PS expressing cells and cell remnants. Diseased tissues on the other hand have a sustained presence of cell surface expressed PS as a result of an imbalance in appearance and clearance of PS expressing cells and cell remnants. Cell surface expressed PS is therefore a useful target for Molecular Imaging and Targeted Drug Delivery (TDD) strategies. This review addresses PS and its binding ligands with potential applications in diagnosis and treatment of a variety of diseases including cardiovascular diseases and cancer.
PS-asymmetry of the plasma membrane

PS is ubiquitously present in prokaryotic and eukaryotic cells and constitutes about 2–10% of total cellular lipids depending on species and cell type. PS synthetic pathways differ between bacteria, yeast and mammals [4]. Mammalian cells synthesize PS predominantly by converting enzymatically phosphatidylcholine (PC) and phosphatidylethanolamine (PE) through a serine exchange reaction. The enzymes PS-synthase 1 (substrate PC) and PS-synthase 2 (substrate PE) catalyze the conversion and are present in the endoplasmatic reticulum (Fig. 1).

PS appears to be crucial to the cell and, as such, is produced by different biosynthetic routes that can compensate each other to maintain a certain minimal level of PS in case one route fails [5, 6]. Cellular PS is non-randomly distributed through several transport mechanisms including vesicular transport and lipid-transfer protein mediated lipid-exchange between juxtapositioned bilayers [7]. Once present in the PM it is subject to the action of the aminophospholipid transporter (APLT) which translocates PS rapidly from the exoplasmic to the cytoplasmic leaflet if PS appears in the exoplasmic leaflet. APLT also translocates PE albeit at a lower rate [8]. The aminophospholipids are thus moved across the bilayer against their gradient and the energy required for translocation is derived from hydrolysis of ATP. APLT appears to be a member of the family of P4 type ATPases, a class of ATPases that mediate ATP-dependently the transbilayer movement of phospholipids [9]. APLT activity is present in erythrocytes, platelets and nucleate cells [10]. In the latter APLT resides in the PM and in trans-Golgi and Golgi derived secretory vesicles. PS asymmetry, once established, is a relatively stable steady state, and APLT activity is required again if disturbances caused by for example membrane fusion processes during endo- and exocytosis occur. It has been shown that inhibition of APLT activity only results in a slow rate of PS exposure [11] indicating that PS asymmetry of the PM is of importance to cell homeostasis.

Cell surface expression of PS

Certain conditions may induce cells to release their PS asymmetry of the PM. For example aging of erythrocyte, activation of platelets and apoptosis are accompanied by a sustained appearance of PS in the exoplasmic leaflet [12, 13]. As indicated above, inhibition of APLT is insufficient to cause rapid and sustained cell surface exposure of PS. An additional mechanism is required to achieve a steady state level of PS in the exoplasmic leaflet. Current main hypothesis describes a scramblase activity to be responsible for PS appearance at the cell surface.

Scramblase translocates phospholipids bidirectionally over the two leaflets of the PM thereby collapsing PS asymmetry. Scrambling is rapid, ATP-independent and non-selective for phospholipid species and it causes randomization of the phospholipids over the two membrane leaflets. Scramblase has been demonstrated to operate in erythrocytes [14], activated platelets [15] and apoptotic cells [16]. Several studies have tried to identify the protein(s) that scramble the phospholipids of the PM. Isolation and reconstitution experiments delivered the protein phospholipid scramblase 1 (PLSCR1), which is the most serious candidate up to now [17]. Closer inspection, however, casted doubt because cells were able to scramble PM phospholipids in the absence of PLSCR1 [18] and six
different cell lines showed a lack of correlation between the level of PLSCR1 expression and the capacity to externalize PS during apoptosis [19]. No other candidates are proposed as yet indicating complexity of phospholipid scrambling and, likely, diversity in scrambling mechanisms. The latter is illustrated by the finding that platelets of a patient with Scott syndrome fail to express PS upon Ca^{2+}-ionophore treatment (a trigger for healthy platelets to expose PS) whilst Scott B-cells normally translocate PS to the cell surface upon execution of apoptosis [20].

Recently an alternative hypothesis was postulated that describes PS externalization as part of membrane repair mechanisms that start to operate during apoptosis and involve fusion of lysosomes with PM [21]. This hypothesis does not require the action of a scramblase protein.

Translocation of PS to the PM exoplasmic leaflet proceeds without compromising the barrier function of the PM. Once in the exoplasmic leaflet PS may participate in a variety of processes depending on type and localization of the PS exposing cell. Circulating erythrocytes for example gradually express PS during aging. PS at the erythrocyte surface functions as an ‘eat me’ signal towards the reticuloendothelial system, which clears the PS tagged erythrocytes from the circulation by phagocytosis [22]. Platelets can participate in hemostatic and thrombotic processes and while doing so can expose PS at their surface. The PS expressing surface catalyzes coagulation reactions that culminate in the formation of thrombin, which subsequently stabilizes the platelet thrombus by generation of fibrin [23]. Activated macrophages that are engaged to engulf dying cells expose PS at their surface. Inhibition of PS exposure greatly impairs phagocytic capacity of the activated macrophage [24]. Vaccinia virus presents PS at the viral membrane to activate PS dependent macropinocytosis with subsequent infection of the host cell [25]. Macrophages and fibroblasts that are infected with Pichinde Virus express PS at the cell surface [26].

The most important and abundant cellular process that is accompanied by cell surface expression of PS is apoptosis, a biochemically regulated process of cell suicide [27]. Firstly described for apoptotic lymphocytes [13] PS exposure is now appreciated as a ubiquitous phenomenon of apoptosis that is independent of cell type and cell death inducing trigger [28] and that is phylogenetically conserved [29]. PS on the surface of an apoptotic cell is one of the most important ‘eat me’ flags that not only triggers engulfment but also activates signaling pathways that control cholesterol efflux and expression of anti- and pro-inflammatory cytokines [30]. In addition, PS on the apoptotic cell surface is involved in regulation of immune response towards antigens of the apoptotic cell [31]. Cells that die by executing a non-apoptotic cell death program also activate a machinery that drives cells surface expression of PS [32] indicating that PS expression is an important phenomenon in dealing with cell death in the context of the multicellular organism. Recognition and engulfment of PS expressing cells are extremely efficient in healthy tissues, which therefore contain, if any, a low steady state level of PS expressing cells. Pathologies can change drastically the balance between appearance and clearance of PS expressing cells towards a sustained presence of PS expressing cells and cell remnants such as apoptotic bodies and cell derived microparticles in diseased tissue. As such surface expressed PS is potentially an informative biomarker for diagnosing disease and evaluating efficacy of therapy. In addition cell surface expressed PS may serve as a target for TDD strategies to deliver therapeutic compounds specifically to diseased tissue.

**PS binding ligands**

In order to exploit fully the potential of PS as a target for Molecular Imaging (diagnosis) and TDD (therapy) ligands should be available that bind selectively and with high affinity to cell surface expressed PS in the complexity of the multicellular organism. To date a variety of PS-binding compounds have been reported in the literature including proteins [2], peptides [33–36] and small chemical entities [37]. As Molecular Imaging agents peptides and small chemical compounds generally have the advantage of being quickly and efficiently cleared from the blood circulation. The signal to background ratio is favorably affected by such kinetics. However, the disadvantage of these compounds concerns their low affinity for binding PS. Proteins on the other hand can exhibit higher affinities for PS but proteins are usually cleared from the blood circulation with slower kinetics. The next sections will highlight in particular the proteins annexin A5, synaptopagmin I and lactadherin with which a body of experience has been build about Molecular Imaging of PS expressing cells in vivo in animal models employing a variety of imaging modalities. To date annexin A5 is the only protein that has been used to visualize PS expressing cells in patients using nuclear imaging. Key characteristics of these three proteins are summarized in Table 1.

**Annexin A5**

Annexin A5 was discovered as an anticoagulant protein of vascular tissue [38]. It is a non-glycosylated single chain protein that belongs to the annexin super-gene family. Its polypeptide is organized in an N-terminal tail with a C-terminal core containing four domains that form the annexin-core, a slightly bent surface with a convex shape that interacts with the PS containing phospholipid
membrane [39, 40]. PS binding of annexin A5 is Ca\(^{2+}\)-dependent. Ca\(^{2+}\)-ions bind to the annexin core surface at type II Ca\(^{2+}\)-binding sites [41] and form the prime contact by coordinating carbonyl and carboxyl groups of the protein and phosphoryl moieties of the glycerol backbone of membrane phospholipids [40]. The domains are composed mainly of \(\alpha\)-helices and the Ca\(^{2+}\)-binding sites protrude as loops (Fig. 2). The overall PS binding affinity arises from a collaboration between the Ca\(^{2+}\)-binding sites of the four domains with a dominant role for domain 1 [42]. The complex structure/function relationship of annexin A5 and PS binding has so far prevented the generation of smaller fragments or mimetics that interact with PS with comparable binding affinity. In solution annexin A5 is present as monomer but once bound to PS-expressing membrane three monomers build a trimer by protein–protein interaction and trimers assemble in a two-dimensional lattice covering the PS expressing surface by trimer-trimer interactions [43]. The two-dimensional protein network of annexin A5 at a PS expressing cell surface drives internalization of annexin A5 [44]. Syntaptotagmin I and lactadherin have reported properties neither of two-dimensional crystallisation on the phospholipid surface nor of internalization via the PS portal of cell entry. Annexin A5 binds PS containing membranes with a \(K_d\) in the range of 0.1–2 nM (Table 1).

Synaptotagmin I

Synaptotagmin I is a synaptic vesicle membrane protein with a short N-terminal intravesicular sequence, a single transmembrane region, and a cytoplasmic region containing two domains with homology to the C2-domain of Protein Kinase C [45–47]. It functions intracellularly as Ca\(^{2+}\)-sensor to mediate synaptic vesicle fusion upon rise of cytoplasmic Ca\(^{2+}\)-levels. Phospholipid binding of syntaptotagmin I is mediated by its C2-domains which bind preferentially the negatively charged phospholipids PS and phosphatidylinositol [48, 49]. PS binding can be exhibited by a single C2 domain as was shown for the first C2 domain (C2A) that was expressed recombinantly by \(E.\ coli\) [37]. The C2A-domain is composed of stable eight-stranded-sandwiches with flexible loops emerging from the top and bottom [48]. These loops bind Ca\(^{2+}\) [50] and acquire subsequently a positive electrostatic potential that becomes attracted by negatively charged phospholipid membranes (Fig. 2) [51]. The C2B domain can bind phospholipids Ca\(^{2+}\)-independently [52]. The dissociation constant (\(K_d\)) of syntaptotagmin I binding to PS is within the 15–40 nM-range (Table 1).

Lactadherin

Lactadherin is a glycosylated protein that was firstly discovered as a component of milk fat globule membranes [53]. It contains an EGF-like domain harbouring an RGD sequence that mediates interaction with the integrin receptors \(\alpha_v\beta_3/5\). At the C-terminal end of the RGD-containing EGF-like domain reside two C domains bearing homologies with the C1 and C2 domains of blood coagulation factors V and VIII. Lactadherin functions as bridging molecule facilitating phagocytosis of dying cells [54, 55]. The RGD-motif interacts with integrin receptors on the surface of phagocytes and the C2-like domain binds in a Ca\(^{2+}\)-independent manner with cell surface expressed PS [56]. The C2-domain of lactadherin contains a \(\beta\)-barrel core with

Table 1: Key characteristics of the PS binding proteins annexin A5, syntaptotagmin I and lactadherin

| Protein          | MW (kD) | Source                                      | PS-binding                          | \(\text{Ca}^{2+}\)-required | \(K_d\) (nM) |
|------------------|---------|---------------------------------------------|-------------------------------------|----------------------------|-------------|
| Annexin A5       | 36      | Human, expressed in \(E.\ coli\) [70]       | Domain                              | Yes                       | 0.1–2       |
| Synaptotagmin I  | 65      | Human, C2A domain expressed in \(E.\ coli\) [83] | Domain\(^a\)                        | Yes                       | 15–40       |
| Lactadherin      | 47      | Bovine, purified from milk [110]            | Domain\(^a\)                        | No                        | 3–4         |

\(^a\) Despite similar nomenclature, the C2-domains of syntaptotagmin I and lactadherin do not share sequence homology.
protruding hydrophobic residues that interact Ca\(^{2+}\)-independently with PS (Fig. 2). The C2-domain of lactadherin shows no significant homology with the C2A domain of synaptotagmin I. Interestingly lactadherin binds PS in a stereo-specific manner [57]. Stereo-specificity has not been observed for the PS binding proteins synaptotagmin I and annexin A5. Lactadherin binds to PS containing membranes with a K\(_d\) ranging from 2 to 4 nM (Table 1).

Other PS binding proteins

Other PS-binding proteins that have potential to be used as ligands for imaging PS expressing cells include T cell immunoglobulin mucins, \(\gamma\)-carboxyglutamic acid (Gla) containing proteins and antibodies directed against PS. T cell immunoglobulin mucin 1 and 4 (TIM-1, TIM-4), both members of the TIM-family were originally identified as a marker of T cell subsets. TIM-proteins are transmembrane proteins that share an immunoglobulin variable domain containing 6 cysteines, a mucin like domain, a transmembrane domain and a cytoplasmic domain [58]. Both TIM-1 and TIM-4 act as a phagocyte receptor for PS expressed on the apoptotic cell [59]. The immunoglobulin domain binds specifically to PS with a K\(_d\) of approximately 2 nM [60].

Gla-domain containing proteins such as vitamin K-dependent blood coagulation factors bind PS through a Ca\(^{2+}\)-mediated interaction between Gla-residues and PS [61]. Gla-domain containing proteins generally bind PS expressing membranes with a K\(_d\) in the nM range.

Immunization procedures with PS as antigen may generate antibodies against PS [62]. However, in most cases immunization results in the generation of antibodies that recognize plasma proteins bound to PS. To target PS on tumor vasculature, the murine monoclonal antibody 3G4 was generated [63]. It appeared that 3G4 does not bind PS directly but through plasma protein 2-glycoprotein 1 that was bound to PS [64]. Plasma protein 2-glycoprotein 1 binds weakly to anionic phospholipids whereas in presence of 3G4 its affinity for anionic membranes increases significantly.

Molecular Imaging of PS

As described above PS expressing cells and cell remnants accumulate in diseased tissues predominantly as a result of the activation of cell death processes and insufficient clearance of the PS expressing cells. Apoptosis is the major process of cell death and plays a role in a wide range of pathologies [65–69]. Therefore non-invasive and tomographic imaging of surface expressed PS has gained interest not only in basic and translational research but also in various clinical disciplines to support diagnosis, localize pathological sites and assess efficacy of therapy. The availability of the PS binding ligand annexin A5 has boosted research and development of Molecular Imaging of PS. To date imaging studies in animal models have been carried out predominantly with various labeled forms of annexin A5. A number of papers have reported about the use of a labeled fusion protein of Glutathion-S-transferase (GST) and the C2A domain of synaptotagmin I. Lactadherin is the less employed one of the three PS binding proteins. Its use has been confined to in vitro studies so far.

Molecular Imaging of PS with Annexin A5

The recombinantly expressed human annexin A5 exhibits PS binding properties identical to annexin A5 purified from human tissue [70]. Availability of recombinant annexin A5 spurred synthesis of a wide range of labeled forms of annexin A5 to accomodate PS imaging with modalities such as optical, radionuclide and magnetic resonance imaging [71, 72]. Annexin A5 is labeled with reporter compounds through chemical coupling mostly to primary amino groups of annexin A5. Since these are also present on the surface of the annexin core amine-based coupling may compromise the PS binding potency [73, 74]. In order to avoid deleterious effects of coupling, annexin A5 variants have been generated for site-directed labeling at the concave side of the molecule using thiol chemistry. Annexin A5 variants have been generated with thiol-linkage sites in extensions of the N-terminus [75, 76] and thiol-linkage sites within the N-terminal tail and the concave side of annexin A5 to which small compounds (chelators of radionuclides [77] and fluorochromes [78]) as well as particles with diameters ranging from 10 to 100 nm (iron oxide nanoparticles [79] and liposomes [80, 81]) have been coupled successfully without impairing PS binding. This so-called ‘second generation’ annexin A5 has improved biodistribution and PS binding properties as compared to amine-labeled wildtype annexin A5.

Molecular Imaging of PS with C2A domain of Synaptotagmin I

Synaptotagmin I is less suitable as a whole molecule for Molecular Imaging because of its transmembrane domain. The soluble PS binding C2A domain was expressed recombinantly by E. coli as a fusion protein with GST. Although the affinity for binding PS is higher for C2A (K\(_d\) = 20–40 nM) as compared to the fusion protein C2A-GST (K\(_d\) = ±115 nM) it was decided to develop C2A-GST as a s ligand because labeling of C2A interfered with PS binding [82]. Labeling of GST-C2A likely occurred predominantly at the GST moiety. C2A-GST can be conjugated to fluorochromes, radionuclides and superparamagnetic iron oxide particles using random chemical linkage while
retaining PS binding property [83, 84]. Whether site-directed chemical linkage will yield a superior PS imaging ligand has not been reported so far.

Molecular Imaging of PS with lactadherin

For PS imaging purposes lactadherin was purified from bovine milk [85]. To date PS imaging with lactadherin has been limited to in vitro studies only. Lactadherin has been coupled to fluorescein isothiocyanate via random chemical linkage to accomodate optical imaging [85]. It is claimed that lactadherin has several advantages as a PS imaging agent over annexin A5 and synaptotagmin I. It binds membranes in a way that is proportional to PS content and independent of both phosphatidylethanolamine and Ca$^{2+}$ [57]. The latter feature is, however, not a benefit in vivo because ionized extracellular Ca$^{2+}$ levels fluctuate around 1 mM which is more than sufficient to promote binding of annexin A5 and C2A-GST to PS expressing membranes. The drawback of lactadherin is its posttranslational modification which precludes expression of functional lactadherin recombinantly in an E. coli system.

The general picture of all imaging studies utilizing annexin A5 and C2A-GST depicts feasibility of PS imaging with non-invasive techniques including optical, radiouclide and magnetic resonance imaging. Non-invasive PS imaging can be employed to accomplish various goals including understanding pathogenesis of cardiovascular diseases such as heart failure [86] and atherosclerosis [87, 88] and evaluating therapeutic in vivo efficacy of drugs such as statins [89] and anti-cancer compounds [90, 91]. Most preclinical non-invasive PS imaging has been carried out with radionuclide and magnetic resonance imaging. Low tissue penetration of photons and autofluorescence of extracellular matrix components have been hampering development of non-invasive optical imaging of PS. Recently near-infrared fluorescent (NIRF) probes and fluorescence mediated tomography (FMT) have been developed rendering non-invasive optical imaging feasible [92]. Second-generation annexin A5 has been coupled to the NIRF probe Vivo-750 via thiol-chemistry and employed successfully to quantify the anti-cancer effect of cytotoxic compounds in a mouse cancer model using non-invasive FMT (Fig. 3).

![3D FMT Imaging](image1)

![FMT Quantification](image2)

**Fig. 3** Non-invasive PS-imaging of efficacy of anti-cancer treatment. Mice were implanted with HT-29 human colorectal adenocarcinoma cells in both upper mammary fat pads, and tumors were allowed to grow for 10 days. To assess the acute induction of apoptosis, animals received a single IP injection of cyclophosphamide (CY; 170 mg/kg). Animals were injected with second generation annexin A5 coupled to Vivo 750 (annexin-Vivo 750, VisEn Medical) 24 h post-CY treatment and imaged 2 h later by FMT 2500 (VisEn Medical) fluorescence tomography. Representative images of treated and untreated HT-29 mice were selected to reflect the mean group tumor fluorescence intensities. (a) 3D FMT images were acquired, and (b) the effects on tumor fluorescence and volume were assessed. Results indicate anti-cancer efficacy of CY in this model. Data and images courtesy of VisEn Medical Inc., Bedford, MA, USA
PS imaging has entered the process of translation from preclinical settings to the clinical arena. Availability of clinical grade recombinant human annexin A5 for labeling with Technetium (Tc-99m) and Single Photon Emission Tomography (SPECT) has catalyzed clinical studies with PS imaging in various patients to assess the significance of PS imaging for diagnosis and rapid evaluation of efficacy of therapy. Table 2 summarizes medical fields in which Molecular Imaging of PS has been interrogated in preclinical models and in patients. These studies show promise for non-invasive imaging of PS to catalyze drug development in preclinical settings and to support diagnosis and evaluation efficacy of treatment shortly after start of therapy in the clinic.

### PS as target for Targeted Drug Delivery (TDD) strategies

TDD is a strategy the goal of which is to treat disease effectively with minimal detrimental side-effects. Such strategies are especially of importance to treatments in which toxic substances are needed to combat disease. TDD is based on the principle of Paul Ehrlich’s ‘Magic Bullet’ which in fact is a therapeutic compound that is guided to the diseased lesion by a targeting function. The targeting function can be an integral part of the therapeutic compound or can be deliberately attached to the drug [93]. Cell surface expressed PS is potentially an attractive target for TDD considering the body of experience with PS as a biomarker for Molecular Imaging [93, 94].

A wide range of diseases may benefit from PS based TDD strategies (see Table 2). In general PS is expressed by dying and dead cells and cell remnants that accumulate in diseased lesions such as atherosclerotic plaques and tumours. In such lesions PS may function to accumulate PS seeking ‘Magic Bullets’ and their therapeutic cargo, which may for example encompass enzymes or cytotoxic substances. Recent experiments indicated that cells, which are not committed to execute cell death, may also express PS on their surface under specific conditions. Endothelial cells of tumour vasculature for example express PS while being alive [95]. Cardiomyocytes that have been submitted to brief ischemia express PS before the apoptotic machinery trespasses the point of no return [96]. These cells can be targeted with therapeutic substances that either kill (tumour endothelial cells) or rescue (stressed cardiomyocytes) the PS expressing cell. Efficient intracellular delivery of the therapeutic substance is then necessary. Annexin A5 has been shown to be internalized into the PS expressing cell as a consequence of its property to form a 2-dimensional lattice on the cell surface [44]. C2A-domain of synaptotagmin I and lactadherin have no reported property of internalization into PS expressing cells.

Recent reports underscored feasibility and applicability of the concept of PS targeting in TDD strategies. Annexin A5 was used as vector to target coagulation and fibrinolytic enzymes to sites of PS expressing cells in the vasculature [97–99]. These PS seeking ‘Magic bullets’ were constructed by molecular fusion [97, 99] or chemical coupling [98] of annexin A5 and the enzyme. Annexin A5 by itself integrates PS targeting and therapeutic function because once bound to PS it blocks the inflammatory and immunomodulatory activities of surface expressed PS [100]. Recently a homodimer of annexin A5 (diannexin) was constructed with the purpose to prolong the blood

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**Table 2** Overview of preclinical and clinical imaging of PS for a variety of diseases and diagnostic purposes

| Medical field          | Diagnostic purpose of PS imaging                                      | References |
|------------------------|-----------------------------------------------------------------------|------------|
|                        | Preclinical studies                                                  | Clinical studies |
| Oncology               | Early assessment of efficacy of therapy                              | [90, 91, 111–114] [115–118] |
|                        | Prognosis of survival                                                | [119]      |
| Cardiology             | Early diagnosis of heart failure                                     | [66, 86, 120–123] [105] |
|                        | Early assessment of cardiac toxicity                                 | [124]      |
|                        | Assessment of cardiac ischemia/reperfusion injury                    | [125–127] [104] |
| Vascular medicine      | Identification of unstable atherosclerotic plaque                    | [128, 129] [130] |
| Orthopaedics           | Assessment of infection of protheses                                 | [131]      |
| Organ transplantation   | Allograft rejection                                                  | [132] [120, 133] |
| Gastroenterology       | Assessment of efficacy of therapy in Crohn’s disease                 | [134]      |
| Neurology              | Identifying regions of cerebral injury                               | [135, 136] [137, 138] |
| Ophthalmology          | Assessment of retinal neurodegeneration                              | [139, 140] |
| Autoimmune diseases    | Identifying regions of rheumatoid arthritis                          | [141]      |
| Metabolic diseases     | Measurement of β-cells apoptosis                                     | [142]      |
circulation time and, hence, to increase therapeutic efficacy of annexin A5 to attenuate ischemia/reperfusion induced injury of organs [101].

PS has also been targeted with the antibody 3G4 (bavituximab), which binds with high affinity to complexes of PS-2-glycoprotein I and PS. 3G4 was used therapeutically as adjuvant therapy in viral infections in which PS surface expression is essential to successful infection [26] and in mouse models of cancer, which have tumour vasculature with PS expressing endothelial cells [102, 103].

Diannexin and bavituximab are the first PS-targeting agents that have entered clinical trials to demonstrate therapeutic activity in patients with kidney transplants, chronic hepatic C virus and HIV and cancer (http://clinicaltrials.gov).

Conclusion and future perspectives

PS is one of the most prominent and ubiquitous fingerprints of cells in diseased tissues and, therefore, an attractive target for Molecular Imaging and translation into clinical applications. Its ubiquity has both advantages and disadvantages. On the one hand a broad spectrum of diseases can be imaged with a single compound but on the other hand ubiquity is accompanied by reduced specificity requiring additional diagnostic steps in order to differentiate. This is exemplified by reports that show that annexin A5 accumulation in the heart of a patient can be the result of acute myocardial infarction [104], ongoing heart failure [105], an intracardiac tumour [106] and infection [107]. Specificity can be increased by more accurate anatomic mapping of the sites where annexin A5 accumulates for example by combining imaging technologies such as SPECT/CT and PET/CT. Development of imaging technology goes in the direction of pin-pointing location of radioisotopes in the human body. More accurate localisation of PS expressing cells will increase the value of PS as a diagnostic biomarker.

PS ubiquity clearly affects TDD aiming at PS. As with Molecular Imaging ubiquity has the advantage of broad application of the PS-TDD concept. Differentiation can be accomplished by tuning the therapeutic cargo of the PS seeking ‘Magic Bullet’ for a specific disease. The disadvantage potentially resides within the side-effects of treatment. No clear picture exists as yet about therapeutic efficacy in relation to undesired side-effects of PS seeking ‘Magic Bullets’. Future studies are necessary in order to fully appreciate the value of PS as a target for TDD strategies to treat diseases.

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