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REVIEW

Annexin A2 complexes with S100 proteins: structure, function and pharmacological manipulation

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Annexin A2 (AnxA2) was originally identified as a substrate of the pp60v-src oncoprotein in transformed chicken embryonic fibroblasts. It is an abundant protein that associates with biological membranes as well as the actin cytoskeleton, and has been implicated in intracellular vesicle fusion, the organization of membrane domains, lipid rafts and membrane-cytoskeleton contacts. In addition to an intracellular role, AnxA2 has been reported to participate in processes localized to the cell surface including extracellular protease regulation and cell-cell interactions. There are many reports showing that AnxA2 is differentially expressed between normal and malignant tissue and potentially involved in tumour progression. An important aspect of AnxA2 function relates to its interaction with small Ca^{2+}-dependent adaptor proteins called S100 proteins, which is the topic of this review. The interaction between AnxA2 and S100A10 has been very well characterized historically; more recently, other S100 proteins have been shown to interact with AnxA2 as well. The biochemical evidence for the occurrence of these protein interactions will be discussed, as well as their function. Recent studies aiming to generate inhibitors of S100 protein interactions will be described and the potential of these inhibitors to further our understanding of AnxA2 S100 protein interactions will be discussed.

LINKED ARTICLES

This article is part of a themed section on Annexins VII Programme. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-7

Abbreviations

AnxA2, annexin A2; Plgn, plasminogen; tPA, tissue plasminogen activator

Tables of Links

TARGETS	
Catalytic receptors[a]	**Enzymes**[b]
TLR4	PKC
	Plasminogen (Plgn)
	RAGE
	Src tyrosine kinase
	Tissue plasminogen activator (tPA)

LIGANDS	
ATP	Histamine
Bepridil	Ketoconazole
cAMP	Trifluoperazine
Forskolin	Von Willebrand factor
Heparin	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

AnxA2 structure

Like all annexins, AnxA2 is a slightly curve-shaped protein with a convex and a concave side. It consists of a highly conserved core domain of four homologous repeats of 70–80 amino acids called the annexin repeats and a unique 30-amino-acid long N-terminal 'head domain' (which is also referred to as tail domain or N-terminal interaction domain in some literature) (Waisman, 1995; Gerke *et al.*, 2005). The core domain region, encompassing residues 31–338, has binding sites for calcium, phospholipids, heparin and F-actin. Although the core domain is conserved among the annexins, subtle differences between them have been noticed. For example, the AnxA2 core domain is subtly different in its Ca^{2+} sensitivity in Ca^{2+}-dependent membrane interactions (Drucker *et al.*, 2013). Thus, different annexins may have different functions in the cell.

The head domain of AnxA2 contains a number of features relatively unique to this particular annexin. The first 12 residues of the head domain constitute the binding site for S100A10, a member of the S100 protein family (Johnsson *et al.*, 1986; 1988). This region also encompasses a binding site for tissue plasminogen activator (tPA) mapped to residues 7–12 (Johnsson *et al.*, 1988; Hajjar *et al.*, 1998) and a nuclear export signal (NES) mapped to residues 3–13 (Eberhard *et al.*, 2001). Residues Tyr^{23}, Ser^{11} and Ser^{25} of AnxA2 can be phosphorylated by Src family tyrosine kinases and serine kinases respectively (Gould *et al.*, 1986; Khanna *et al.*, 1986; Powell and Glenney, 1987; Jost and Gerke, 1996).

The interaction of AnxA2 with S100 proteins

Perhaps one of the most clearly defined features that characterize AnxA2 is its capacity to interact with members of the S100 protein family to yield so-called heterotetrameric complexes, consisting of an S100 protein dimer and two AnxA2 proteins ($(S100AXX\text{-}AnxA2)_2$). S100 proteins are a group of small Ca^{2+}-binding proteins with molecular weight of 10–12 kDa (Donato, 1999; 2003). With the exception of S100A10, they contain Ca^{2+}-binding EF-hand motifs, and are regarded as the largest family grouping within the EF-hand protein superfamily. Rather uniquely, compared with other EF-hand proteins, S100 monomers contain two different EF hands with distinct affinities for calcium: a canonical C-terminal EF hand ($K_D \approx$ 10–50 μM) and a pseudo-canonical N-terminal EF hand ($K_D \approx$200–500 μM). S100 proteins always function as dimers, mostly homodimers, but sometimes heterodimers: S100A1/B, S100A8/A9, S100A1/A4 and S100A1/P (Odink *et al.*, 1987; Duda *et al.*, 1996; Tarabykina *et al.*, 2000; Wang *et al.*, 2004). Ca^{2+} binding induces a conformational change in the S100 proteins due to repositioning of helix III from a near antiparallel position to helix IV to a nearly perpendicular position. Thus, a compact and closed conformation opens up and exposes a large hydrophobic area which is capable of recognizing and binding potential targets (Malashkevich *et al.*, 2008). S100A10 is unique among S100 proteins in that it is locked in a permanently open conformation, comparable to the Ca^{2+}-bound configuration of the other S100 proteins. Many S100 proteins play a role in cancer prognosis or progression (Schlagenhauff *et al.*, 2000; Davies *et al.*, 2002; Cross *et al.*, 2005; Vimalachandran *et al.*, 2005; De Petris *et al.*, 2009) and some of them are suggested as biomarkers to certain types of cancer (Hamberg *et al.*, 2003; Nedjadi *et al.*, 2009; Tsuna *et al.*, 2009).

The classic AnxA2-binding S100 protein is S100A10, which was identified as a binding partner almost 30 years ago (Erikson *et al.*, 1984; Gerke and Weber, 1985a,b). Binding to S100A10 occurs at the helical AnxA2 N-terminus (Glenney *et al.*, 1986; Johnsson *et al.*, 1988; Kube *et al.*, 1992; Rety *et al.*, 1999). The AnxA2 N-terminus is accommodated in the free hydrophobic space between helix III and helix IV of the S100A10 dimer (Rety *et al.*, 1999) (Figure 1). Interaction with the AnxA2 N-terminus appears sufficient for binding since proteolytic removal of this N-terminus from purified AnxA2 (cleaved at Gly14) (Johnsson *et al.*, 1988) or deletion of residues 1–14 from recombinant AnxA2 (e.g. Semov *et al.*, 2005) results in a complete loss of the interaction with S100A10. Removal of the first methionine of the primary AnxA2 translation product as well as acetylation of the serine at position 2 is necessary for AnxA2 binding to S100A10 (Johnsson *et al.*, 1988; Becker *et al.*, 1990; Konig *et al.*, 1998; Nazmi *et al.*,

2012). In addition to the acetyl group, specific hydrophobic residues are crucial for binding with S100A10 (Becker *et al.*, 1990; Rety *et al.*, 1999).

Recent studies showed that in addition to S100A10, three other S100 proteins, S100A6, S100A4 and S100A11, are also able to bind to AnxA2 (Table 1). Various techniques have been employed to investigate the binding between these S100 proteins and AnxA2. Isothermal titration calorimetry of 16 different S100 proteins with the AnxA2 N-terminus identified S100A10 and S100A11 as binding partners (Streicher *et al.*, 2009). The interaction between S100A11 and AnxA2 was also demonstrated by nuclear magnetic resonance (NMR), isothermal titration calorimetry and immunoprecipitation (Rintala-Dempsey *et al.*, 2006). Although not observed using isothermal titration calorimetry, S100A4 can accommodate the AnxA2 N-terminus based upon nuclear NMR and immunoprecipitation evidence (Semov *et al.*, 2005). The NMR studies indicated that Glu^{6}, Asp^{10}, Leu^{42}, Phe^{45}, Ile^{82}, Phe^{89} and Pro^{94} in S100A4 could be involved in the binding with AnxA2 and these amino acids are in similar positions to key amino acids in S100A10 involved in the binding to AnxA2 (Glu^{5}, Glu^{9}, Phe^{38}, Phe^{41}, Leu^{78}, Tyr^{85} and Met^{90} in S100A10) (Semov *et al.*, 2005). Finally, an AnxA2 complex with S100A6 was identified using affinity chromatography and immunoprecipitation methods (Zeng *et al.*, 1993) and further confirmed by biochemistry and spectrometry methods (Filipek *et al.*, 1995; Nedjadi *et al.*, 2009). Given the similarity of the calcium-bound conformations of S100A4, S100A6 and S100A11 to the S100A10 conformation, it may be argued that AnxA2 is accommodated in a similar fashion in each of these S100 proteins.

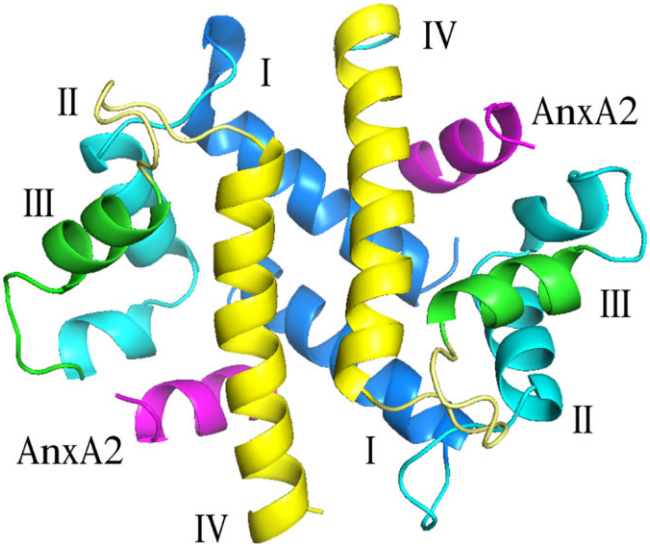

Figure 1

Structure of S100A10 binding with annexin A2 peptide displayed as ribbon diagram. S100 proteins are coloured blue green yellow while the AnxA2 N-terminus is coloured magenta (PDB: 1BT6) (Rety *et al.*, 1999).

Several studies have investigated the affinity of the AnxA2 N-terminus for S100 proteins. Using nuclear magnetic resonance techniques, a dissociation constant of 3.3 ± 0.6 μM was derived for the binding of AnxA2 to S100A11 by titrating an AnxA2 N-terminus peptide into calcium-bound S100A11 (Rintala-Dempsey *et al.*, 2006). Isothermal titration calorimetry experiments have also been used to determine the dissociation constant of this interaction (Streicher *et al.*, 2009). It was found that the binding isotherm could not be fitted to the simplest binding model, but fitted into a sequential binding model suggesting that the interaction involves non-symmetric binding to the two AnxA2 peptides: one binding site on the dimer needs to be occupied before the second binding event can take place. Thus, two dissociation constants were determined for the binding of the AnxA2 N-terminus to S100A11: 1.7 ± 1.2 μM and 9.2 ± 1.9 μM. A very similar scenario applied to the binding of the AnxA2 N-terminus to S100A10 albeit that the two sequential binding events appeared to have identical dissociation constants of 0.5 ± 0.4 μM (Streicher *et al.*, 2009). A comparable dissociation constant of 1.3 ± 0.3 μM was measured independently for this interaction using equilibrium dialysis and fluorescence resonance transfer techniques (Li *et al.*, 2010) and for the binding of full-length AnxA2 to S100A10 (Nazmi *et al.*, 2012).

Table 1

S100 proteins known to bind AnxA2

AnxA2 targets	IP	X-ray	ITC	Biochemistry method	Spectrometry or chromatography method
S100A10	Erikson *et al.*, 1984 Gerke *et al.*, 1985a Gerke *et al.*, 1985b	Rety *et al.*, 1999	Streicher *et al.*, 2009	Johnsson *et al.*, 1988 Li *et al.*, 2010 Streicher *et al.*, 2009	–
S100A4	Semov *et al.*, 2005	–	–	–	Semov *et al.*, 2005
S100A6	Zeng *et al.*, 1993 Filipek *et al.*, 1995 Nedjadi *et al.*, 2009	–	–	Filipek *et al.*, 1995	Zeng *et al.*, 1993 Filipek *et al.*, 1995 Nedjadi *et al.*, 2009
S100A11	Rintala-Dempsey *et al.*, 2006	–	Streicher *et al.*, 2009 Rintala-Dempsey *et al.*, 2006	–	Rintala-Dempsey *et al.*, 2006

IP, immunoprecipitation; ITC, isothermal titration calorimetry; X-ray, crystallography.

The structure of the full AnxA2-S100 complex

In appreciating the well-established binding mode of the AnxA2 N-terminus to S100A10, the structure of the full complex is more speculative. Modelling the complex between the S100A10 dimer and the AnxA2 N-terminus with the solved AnxA2 core domain suggests two plausible configurations. In one model, the S100A10 dimer bridges two AnxA2 molecules arranged in opposite orientation whereas the second model predicts the S100A10 dimer to sit on top of two AnxA2 molecules arranged side by side (Sopkova-de Oliveira Santos *et al.*, 2000). It is extremely difficult to ascertain which of these models would prevail *in vivo*; however, individually they can explain various functions of the AnxA2 protein and therefore both conformations may exist. In terms of how these various conformations are regulated, it is of interest that the interaction of AnxA2 with the S100A10 dimer takes place at the very end of the protein, leaving a loop region between this S100A10 recognition region and the AnxA2 core domain. Flexibility in this loop may allow the one or the other conformation. Electron microscopy data of membrane bridges at pH 7.4 in the presence of Ca^{2+} suggest that the dimer of S100A10 be located in the centre of the protein density, with one molecule of AnxA2 facing the bilayer on each side (Lambert *et al.*, 1997). However, AnxA2 can also move around S100A10 as a hinge to acquire a more open conformation where the S100A10 subunit is held away from the phospholipid bilayer (Lambert *et al.*, 2004; Menke *et al.*, 2004; Illien *et al.*, 2010), compatible with the suggested models (Sopkova-de Oliveira Santos *et al.*, 2000). A third more stretched conformation has been observed experimentally at mild acidic pH in the absence of Ca^{2+} (Illien *et al.*, 2010). Cellular studies indicated that acidic pH can support the membrane binding of the $(S100A10\text{-}AnxA2)_2$ heterotetramer complex and it is perhaps this stretched conformation that is responsible for this (Monastyrskaya *et al.*, 2008). Interestingly, the putative hinge region of the annexin head domain is subject to phosphorylation and phosphorylation events may also regulate the specific loop architecture and conformation of the tetramer (Grindheim *et al.*, 2014).

The cellular complex of AnxA2 and S100 proteins

The ratio of monomeric AnxA2 to $(S100A10\text{-}AnxA2)_2$ can vary widely, and the differences in ratio are due to coordinated expression of AnxA2 and S100A10 (Munz *et al.*, 1997) as well as post-translational control (Puisieux *et al.*, 1996). Interfering with the expression of one of the partners in the $(S100A10\text{-}AnxA2)_2$ complex affects the expression of the other partner, indicating their intimate relationship *in vivo*. In most reports, knockdown of AnxA2 affects the levels of S100A10; however, the reverse has also been observed in some cases and thus the 'direction' of the regulatory effect seems to differ between cell types. For example, in endothelial cells, knockdown of AnxA2 not only results in the disappearance of AnxA2 but also in that of S100A10, while knockdown of S100A10 does not affect expression of AnxA2

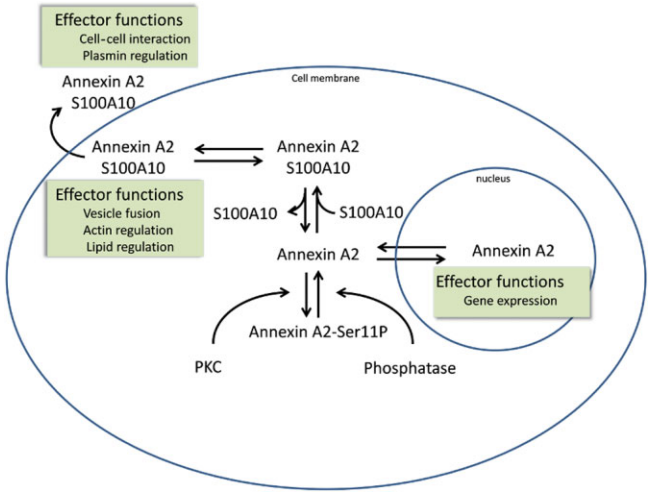

Figure 2

Simplified diagram to illustrate some aspects of the cellular regulation of AnxA2 by S100 protein interactions.

(Brandherm *et al.*, 2013). In complex with S100A10, AnxA2 may protect S100A10 from being rapidly polyubiquitinated and degraded (He *et al.*, 2008). Interestingly, the residues 86–95 subject to ubiquitination of S100A10 are the residues responsible for binding with the AnxA2 N-terminal, suggesting that once ubiquitinated, S100A10 may not bind AnxA2 anymore. The amount of S100A10 in the cell would thus dictate the amount of the $(S100A10\text{-}AnxA2)_2$ complex in the cell (Figure 2). This may be important to determine the intracellular fate of AnxA2. Although AnxA2 itself can associate with cellular membranes (Zobiack *et al.*, 2001), S100A10 binding increases the Ca^{2+} sensitivity of AnxA2 and its capacity to bind membranes and (submembranous) F-actin (Ikebuchi and Waisman, 1990; Harder and Gerke, 1994; Filipenko and Waisman, 2001; Monastyrskaya *et al.*, 2007). Depletion of S100A10 by RNA silencing or phosphorylation on Ser^{11} on AnxA2 (which inhibits interaction with S100A10) disrupts the membrane association of AnxA2 (Regnouf *et al.*, 1995; Deora *et al.*, 2004). Similar observations have been made for S100A6 which has also been proposed to interact with AnxA2. Depletion of S100A6 from pancreatic cancer cells was accompanied by diminished levels of membrane AnxA2 associated with a pronounced reduction in the motility of pancreatic cancer cells (Nedjadi *et al.*, 2009). Under certain conditions of stress, AnxA2 can become expressed on the cell surface, in a mechanism that requires the interaction with S100A10 as well as phosphorylation of AnxA2 on tyrosine at position 23 (Deora *et al.*, 2004).

Sequestration of AnxA2 by S100A10 in the cytosol also prevents its nuclear localization (Eberhard *et al.*, 2001). In prostate cancer cells, monomeric AnxA2 can localize to the nucleus where it acts as negative regulator of cell proliferation (Liu *et al.*, 2003). Phosphorylation of AnxA2 may be important for its nuclear localization (Chiang *et al.*, 1996; Eberhard *et al.*, 2001). AnxA2 contains a functional NES sequence at the N-terminal which allows export via the Ran/exportin-mediated export pathway (Eberhard *et al.*, 2001). This

sequence overlaps with the binding site of AnxA2 with S100A10.

As mentioned above, loss of S100A10 has also been observed to affect the levels of AnxA2, in particular in studies in which S100A10 was removed by genetic deletion. In S100A10 knockout mice, the AnxA2 level decreased in spleen, kidneys, lungs and liver, but was not affected in intestine (Madureira *et al.*, 2012), suggesting that S100A10 could stabilize and regulate the level of AnxA2. However, studies in nociceptor neurons indicated that genetic deletion of S100A10 did not affect AnxA2 levels (Foulkes *et al.*, 2006).

Functions associated with the AnxA2-S100 complex

Biochemical reconstitution experiments, mouse genetic deletion models and RNA interference studies have yielded much information on the effector functions of individual annexins and S100 proteins, including AnxA2 and S100A10. It is beyond the scope of this review to discuss all the evidence and the reader is referred to recent excellent reviews in this area (Gerke and Moss, 2002; Rescher and Gerke, 2004; Gerke *et al.*, 2005; Kwon *et al.*, 2005; Flood and Hajjar, 2011; Madureira *et al.*, 2011; Bharadwaj *et al.*, 2013; Luo and Hajjar, 2013). However, a few recent relevant examples are cited here to provide an indication of the effector function of $(S100A10\text{-}AnxA2)_2$ and related complexes in the cell. It is perhaps useful to consider these in relation to the two conformational models cited above. While it is very difficult to ascertain precisely which model applies to a particular cellular context, in a broad simplification one could say that in one conformation, the 'opposite conformation', the tetramer can bring together different cell membranes, while in the other conformation, the 'lateral conformation', the tetramer can act as a platform for association with other proteins. This classification should, however, not be taken as absolute, since the former conformation could accommodate additional proteins and the latter can serve to juxtapose membranes.

The former conformation points to a role in membrane trafficking, secretory or endocytic processes. A recently presented example shows that the $(S100A10\text{-}AnxA2)_2$ complex is involved in the secretion of von Willebrand factor (vWF), which is stored in the Weibel–Palade bodies (secretory granules) of endothelial cells (Knop *et al.*, 2004; Brandherm *et al.*, 2013). It is normally released by agonists that raise intracellular Ca^{2+} or cAMP levels and a functional $(S100A10\text{-}AnxA2)_2$ complex is required for the forskolin-induced, cAMP-dependent release of vWF (Knop *et al.*, 2004; Brandherm *et al.*, 2013). Forskolin triggers dephosphorylation of AnxA2 (Borthwick *et al.*, 2007), mediated by a calcineurin-like phosphatase. This stabilizes the $(S100A10\text{-}AnxA2)_2$ complex and promotes vWF release (Brandherm *et al.*, 2013). When the $(S100A10\text{-}AnxA2)_2$ complex cannot form, cAMP-dependent vWF secretion is compromised (Brandherm *et al.*, 2013). At present, it is not clear whether additional protein interactions contribute to the secretion of vWF such as observed in secretory processes in stimulated chromaffin cells. In these cells, AnxA2 directly interacts with S100A10 to form a tetramer at the plasma membrane (Chasserot-Golaz *et al.*, 2005; Umbrecht-Jenck *et al.*, 2010). S100A10 can interact with vesicle-associated membrane protein 2 (VAMP2) which may act as docking factor for S100A10. Prevention of S100A10 binding to VAMP2 inhibits the translocation of annexin-A2 to the plasma membrane. In bronchial epithelial cells, AnxA2 associates with collagen VI and the SNARE proteins SNAP-23 and VAMP2 at secretory vesicle membranes, and as such has been implicated in the collagen VI secretion pathway (Dassah *et al.*, 2014). It is not clear whether this also involves the S100A10 protein interaction.

Localized at the cell surface, AnxA2 has been implicated in cell-cell interactions and cell adhesion. AnxA2 provides a signal for interaction with and phagocytosis of apoptotic cells, most likely via interactions with phosphatidyl serine on the juxtaposed apoptotic cell surface (Fan *et al.*, 2004; Law *et al.*, 2009; Fang *et al.*, 2012). AnxA2 expressed on apoptotic cells themselves binds complement factors as signal for cell-cell interaction and phagocytosis (Leffler *et al.*, 2010; Martin *et al.*, 2012). Furthermore, the $(S100A10\text{-}AnxA2)_2$ complex has been implicated in tight junction maintenance in epithelial MDCK cell monolayers in a model in which AnxA2 is associated with the lipid membrane with the S100A10 dimer bridging two AnxA2 molecules (Lee *et al.*, 2004; 2008). The binding of surface AnxA2 to surface S100A10 also contributes to heterotypic cell-cell interactions between breast tumour cells and microvascular endothelial cells. An AnxA2 molecule present on an opposing cell, such as a breast cancer cell, can bridge to the endothelial cell by interacting with surface-localized S100A10 located on the latter (Myrvang *et al.*, 2013).

A wide range of platform functions of the $(S100A10\text{-}AnxA2)_2$ complex have been suggested. Early research revealed that as well as bridging phospholipid vesicles and binding biological membranes, the $(S100A10\text{-}AnxA2)_2$ complex displays binding and bundling of F-actin (Gerke and Weber, 1985a). This occurs at physiological Ca^{2+} concentrations in the μM range (Ikebuchi and Waisman, 1990; Regnouf *et al.*, 1991). This activity can be specifically inhibited by pre-incubation of F-actin with a nonapeptide to the actin-binding site of AnxA2 at residues 286–294 (Jones *et al.*, 1992). The $(S100A10\text{-}AnxA2)_2$ complex is important for the organization of F-actin at lipid rafts and for the dynamic regulation and remodelling of the actin cytoskeleton (Hayes *et al.*, 2004; 2006). As such, AnxA2 has been implicated in various cellular processes that involve the actin cytoskeleton.

One of the other AnxA2 partners, S100A11, is required for efficient plasma membrane repair which may support the survival of invasive cancer cells (Jaiswal *et al.*, 2014). During cell migration and invasion, cells are exposed to physical stress. Injury to the cell membrane occurring during this process results in entry of calcium into the cell which in turn can trigger recruitment of S100A11 and AnxA2 to the site of injury (Jaiswal *et al.*, 2014). The complex of S100A11 with AnxA2 directs polymerization of cortical F-actin and excision of the damaged part of the plasma membrane thereby resealing the plasma membrane (Jaiswal *et al.*, 2014).

On endothelial cells, the $(S100A10\text{-}AnxA2)_2$ complex has been proposed as endothelial surface platform for tPA and plasminogen (Plgn), aiding the conversion to plasmin.

Several somewhat conflicting models exist to explain the exact contributions of these proteins individually (or as a complex) to the plasmin activation process with either AnxA2 (Cesarman *et al.*, 1994; Hajjar *et al.*, 1994; 1998; Flood and Hajjar, 2011; Luo and Hajjar, 2013) or S100A10 (Kassam *et al.*, 1998; MacLeod *et al.*, 2003; Madureira *et al.*, 2011; Bharadwaj *et al.*, 2013) proposed as the main receptor of tPA and Plgn. Both models implicate the binding of S100A10 and AnxA2 in the regulation of the surface proteases and the fact that genetic deletion of either protein shows roles for both proteins in maintenance of vascular patency, fibrin resolution, cell migration and neoangiogenesis also suggests a very close relationship between them, most likely because they act as a complex in these processes (Ling *et al.*, 2004; Huang *et al.*, 2011; Madureira *et al.*, 2011; Phipps *et al.*, 2011; Surette *et al.*, 2011).

Like the complex of AnxA2 and S100A10, the complex of AnxA2 and S100A4 may also regulate the tPA/Plgn cascade on the cell surface. Addition of S100A4 to umbilical vein endothelial cells stimulated tPA/Plgn on these cells. This stimulation was reversible upon addition of a synthetic peptide based upon the AnxA2 N-terminus, suggesting that a complex between S100A4 and AnxA2 is involved in tPA/Plgn regulation (Semov *et al.*, 2005). This scenario may be relevant when tumour cells produce S100A4 which, once bound to endothelial cells and activating pericellular proteases, can aid the growth of blood vessels into the tumour.

While the F-actin-binding and protease-regulating platform functions of the $(S100A10\text{-}AnxA2)_2$ complex are not clearly defined in structural terms, interactions with the protein AHNAK and SMARCA3 have been solved by protein crystallography. AHNAK, a Hebrew word for 'giant', is a 629 kDa protein involved in membrane repair (Shtivelman *et al.*, 1992; Zhang *et al.*, 2004). A multi-protein complex of $(S100A10\text{-}AnxA2)_2$ and AHNAK is a target of dysferlin, a core protein in wound repairing process of 'injured' epithelial cells (Huang *et al.*, 2007). The minimal binding site of AHNAK protein, $AHNAK^{5654–5673}$, with the $(S100A10\text{-}AnxA2)_2$ complex has been mapped (De Seranno *et al.*, 2006; Rezvanpour *et al.*, 2011) and was used to derive a crystal structure (Ozorowski *et al.*, 2013). The 20-amino-acid length AHNAK peptide binds asymmetrically across the $(S100A10\text{-}AnxA2)_2$ complex. Hydrogen bonding between backbone atoms of ANNAK peptides and $(S100A10\text{-}AnxA2)_2$ and the hydrophobic interaction of ANNAK side chains with S100A10 are the main binding forces responsible for the ternary complex (Ozorowski *et al.*, 2013).

It was recently found that SMARCA3, a protein involved in chromatin remodelling in different nuclear processes in an ATP-dependent manner (Debauve *et al.*, 2008), is also a target of the $(S100A10\text{-}AnxA2)_2$ complex (Oh *et al.*, 2013). The co-crystal structure of SMARCA3 with this complex shows that the binding site is very similar to that of AHNAK peptide: two SMARCA3 peptides symmetrically bind to the $(S100A10\text{-}AnxA2)_2$ complex at the 'back' of S100A10 reaching into a small hydrophobic cavity created by the 'C-terminal' of AnxA2 peptide and helix IV(IV') (Oh *et al.*, 2013). It was observed that the $(S100A10\text{-}AnxA2)_2$ complex can increase the DNA binding affinity of SMARCA3 and help SMARCA3 localize to the nuclear matrix; this would require S100A10 to be present in the nucleus.

A peptide toolbox to study AnxA2-S100 protein interactions

Given the detailed knowledge of the binding interactions between the N-terminus of AnxA2 and S100A10, various groups have reported the use of peptides based upon this N-terminus to perform competition experiments with the aim of disrupting the endogenous complex of the two proteins and understanding its functions. An isolated acetylated synthetic peptide comprising residues 1–14 of AnxA2 can disrupt a preformed complex between S100A10 and a labelled annexin 1–14 peptide (O'Connell *et al.*, 2010). Furthermore, the same peptide also disrupts a preformed full-length $(S100A10\text{-}AnxA2)_2$ complex (Konig *et al.*, 1998). Thus, it is feasible to use synthetic peptides to disrupt endogenous complexes between AnxA2 and S100A10 *in vivo* (Table 2).

Because of their nature, peptide interference studies have largely been confined to scenarios where AnxA2 and S100A10 are localized at the outer face of the plasma membrane, or where the peptide could somehow be introduced into the cells, for example, by microinjection or in patch clamp experiments (Table 2). Very elegant studies have been performed in which the action of an acetylated peptide was compared with a non-acetylated peptide. It is known that the non-acetylated version of AnxA2 (or its N-terminus) binds weakly to S100A10 dimers, therefore it is expected that such a peptide cannot disrupt the endogenous complex (Becker *et al.*, 1990). By studying both peptides in parallel, a convincing argument can be made for or against the involvement of the $(S100A10\text{-}AnxA2)_2$ complex in a cellular process under study. In this way, it was shown that an acetylated version of the annexin N-terminus peptide reduced the volume activation of a chloride current in pulmonary artery endothelial cells, whereas a non-acetylated version of the same peptide did not affect the current, implicating the S100A10 protein interaction with AnxA2 in activation of these ion currents (Nilius *et al.*, 1996). The same strategy has revealed the involvement of the $(S100A10\text{-}AnxA2)_2$ complex in histamine induced secretion of vWF from endothelial cells (Knop *et al.*, 2004).

The AnxA2 N-terminus peptide is able to compete with cell-cell interactions between breast cancer cells and endothelial cells while a scrambled peptide is not (Myrvang *et al.*, 2013). It was observed that AnxA2 is present on the surface of breast cancer cells, and S100A10 on the surface of endothelial cells. Thus, these proteins may function as bridge between these cell types in cell-cell interactions. Similar studies using competing N-terminus peptides indicate that $(S100A10\text{-}AnxA2)_2$ complexes are involved in tight junction assembly between kidney epithelial cells, suggesting a role in cell-cell interactions (Lee *et al.*, 2004).

The adhesion of prostate cancer cells to bone marrow endothelial cell is also inhibited by AnxA2 N-terminus peptides. A putative AnxA2 receptor has been identified on prostate cancer cells, which may aid the cell-cell interaction (Shiozawa *et al.*, 2008).

A peptide based upon the AnxA2 N-terminus, but not a scrambled peptide, has been shown to inhibit neoangiogenesis into Matrigel plugs (Ling *et al.*, 2004), suggesting that protein interactions at the AnxA2 N-terminus participate in

Table 2

Peptide inhibitors used to elucidate the function of annexin A2 protein interactions

Peptide	Test system	Observation	Reference
AA2 (1–14)	Chloride channel activation measured by patch clamp	Reduced by acetylated but not by non-acetylated peptide	Nilius *et al.*, 1996
AA2 (1–14)	Purified AnxA2/S100A10 complex binding to liposomes	Loss of binding to liposomes in the presence of peptide	Konig *et al.*, 1998
AA2 (1–14)	vWF release after micro injection	Reduced by acetylated but not by non-acetylated peptide	Knop *et al.*, 2004
AA2 (1–14)	Formation of epithelial cell tight junctions *in vitro*	Reduced by peptide	Lee *et al.*, 2004
AA2 (1–14)	FGF- and VEGF-driven angiogenesis into Matrigel plug *in vivo*	80% decrease in vascularization by the peptide	Ling *et al.*, 2004
AA2 (7–12)	Pancreatic cancer cell migration	Inhibited by peptide at high concentrations	Diaz *et al.*, 2004
AA2 (1–14)	S100A4-induced, tPA-mediated plasminogen activation on endothelial cells	Inhibited by the peptide	Semov *et al.*, 2005
AA2 (1–12)	Adhesion of embryonic stem cells to annexin A2 *in vitro*	~80% inhibition	Jung *et al.*, 2007
AA2 (1–14)	AnxA2/S100A10 complex formation with CFTR in co-immunoprecipitates	Binding of AnxA2 reduced by acetylated but not by non-acetylated peptide	Borthwick *et al.*, 2007
AA2 (1–12)	Adhesion of prostate cancer cells to endothelial cell monolayer	Reduced by peptide	Shiozawa *et al.*, 2008
AA2 (1–12)	Homing of prostate cancer cells to bone marrow *in vivo* (metastasis)	Reduced by peptide	Shiozawa *et al.*, 2008
AA2 (1–14)	Adhesion of breast cancer cells to endothelial cell monolayers	Reduced by acetylated but not by scrambled peptide	Myrvang *et al.*, 2013

CFTR, cystic fibrosis transmembrane conductance regulator; FGF, fibroblast growth factor.

this process. The peptide may compete with an endogenous $(S100A10\text{-}AnxA2)_2$ complex at the endothelial cell surface, or alternatively it may inhibit directly the interaction between AnxA2 and tPA.

This last scenario illustrates the power of the use of synthetic peptides in elucidating the involvement of the $(S100A10\text{-}AnxA2)_2$ complex in physiological processes but also the problem, since additional interactions are possible (at least in principle) to explain the observations.

Chemical manipulation of the AnxA2-S100 protein interaction

Protein interactions are generally considered not amenable to blockade with small molecules. This is largely because they involve shallow and extensive interfaces with no features that could support effective small molecule binding. However, there are cases of successful targeting of protein interactions. For example, the interaction between Mdm2 and p53, and the interaction between Bcl2 and Bak have both been explored pharmacologically using small molecule inhibitors, which have subsequently shown promise as therapeutic agents (Shangary and Wang, 2008; 2009; Gandhi *et al.*, 2011). It is of interest that both p53 and Bak contain a short helical sequence that docks into a well-defined groove-like feature on the surface of the respective binding partners, which in both cases constitutes a small globular protein. The protein interaction between the AnxA2 N-terminus and S100A10 proteins similarly involves a well-defined and comparatively deep concave binding pocket accommodating a small helical peptide. The AnxA2 N-terminus conceals approximately 660 $Å^2$ of solvent-accessible surface area in the lipophilic pocket of S100A10 (Rety *et al.*, 1999). Most of the binding energy derives from hydrophobic interactions in the innermost portion of the pocket and from charge-enhanced H-bonds with the carboxyls of E5 and E9 of S100A10 (Becker *et al.*, 1990; Rety *et al.*, 1999). Residues V3, I6, L7 and L10 alone displace approximately 430 $Å^2$ of solvent-accessible surface area. This is a size of binding pocket that is within reach of what are commonly considered drug-like molecules (Lipinski, 2004).

A receptor-guided as well as a ligand-guided virtual screening approach was recently used to identify a novel class of small molecules that inhibit the interaction between AnxA2 and S100A10 (Reddy *et al.*, 2011; 2012; 2014) (Figure 3). This virtual screening approach allowed the identification of candidate blockers that were able to dock into the AnxA2-binding site on S100A10 or that mimicked the binding pose of the AnxA2 N-terminus as defined in the complex crystal structure (Rety *et al.*, 1999). Candidate molecules were then screened in a biochemical FRET assay that measured the binding between the AnxA2 N-terminus and

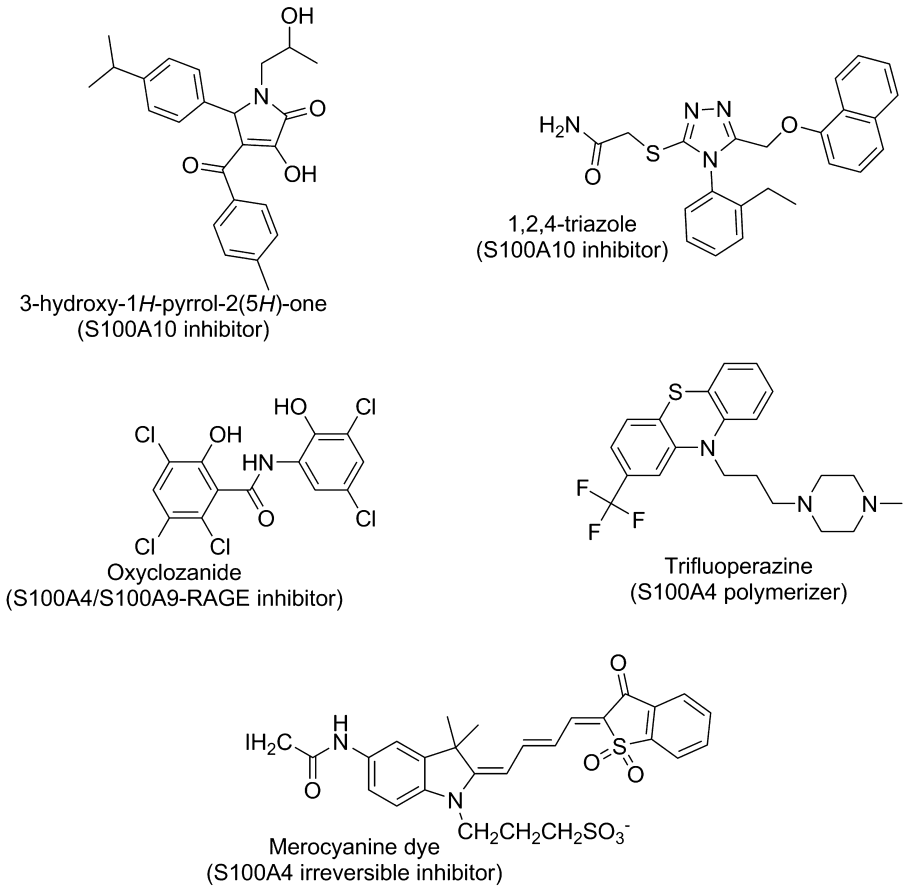

Figure 3
Chemical structures of S100 protein interaction inhibitors.

the S100A10 protein. This identified two classes of compounds: 3-hydroxy-1*H*-pyrrol-2(5*H*)-one analogues and substituted 1,2,4-triazoles as effective blockers of the binding of S100A10 and AnxA2 (Reddy *et al.*, 2011; 2012). The docking suggested that both kinds of inhibitors could bind to three pockets on S100A10 that are normally occupied by an acetyl, valine and leucine moiety on the AnxA2 N-terminus (Reddy *et al.*, 2011; 2012). Selected blockers were also able to inhibit the interaction of the native complex of AnxA2 and S100A10 and some were shown to inhibit the complex inside the cell. These compounds may be used to further elucidate the function of the $(S100A10\text{-}AnxA2)_2$ complex.

The compound withaferin A has been shown to bind to the N-terminus of AnxA2 via covalent bonding to the cysteine residue at position 9 (Ozorowski *et al.*, 2012). This residue is solvent exposed in the $(S100A10\text{-}AnxA2)_2$ complex and withaferin A did not inhibit the protein interaction between the proteins. However, it may inhibit functions of the monomeric form of AnxA2.

In addition to the S100A10 AnxA2 blockers described above, a number of additional S100A4 protein blockers have been described that could conceivably be useful in understanding interactions between S100 proteins and annexins. Merocyanine can covalently bind to Cys^{81} of S100A4 and act as an irreversible inhibitor of the binding of S100A4 to myosin IIA (Garrett *et al.*, 2008). Cys^{81} is a part of the hydrophobic area on S100A4 that could possibly be involved in the binding with AnxA2 (Semov *et al.*, 2005), indicating that interactions with AnxA2 may also be inhibited by this compound. A set of Food and Drug Administration-approved drugs was tested for their ability to inhibit the Ca^{2+}-induced conformational change of S100A4 as determined by a fluorescence increase of the linked biosensor. This identified a number of phenothiazine compounds. Phenothiazines were found to defunctionalize S100A4 by polymerizing the protein (Malashkevich *et al.*, 2010). Other compounds affecting the S100A4 conformation included ketoconazole, bepridil and nicergoline (Garrett *et al.*, 2008). Bepridil can inhibit the myosin IIA filament depolarizing effect of S100A4. It is not known whether compounds like these interfere with the AnxA2-binding properties of S100A4.

Oxyclozanide has been shown to inhibit the interaction between S100A4 and receptors for advanced glycation end products (RAGEs) or toll-like receptor 4 (TLR4) (Bjork *et al.*, 2013). It appears to bind to the homodimeric form or to an S100A4/A9 heterodimer to interfere with the binding with RAGE and TLR4 (Bjork *et al.*, 2013). These interactions are implicated in inflammation and tumour growth (Foell and Roth, 2004; Apetoh *et al.*, 2007; Gebhardt *et al.*, 2008; Bjork *et al.*, 2009) as well as cell matrix invasion (Yammani *et al.*,

2006). Again, it remains to be established whether this compound interferes with the AnxA2-binding properties of S100A4.

Conclusion

The interaction between S100 proteins and AnxA2 plays a role in various processes in the cell. The recent identification of small molecule inhibitors of this interaction, combined with known peptidic inhibitors, will allow further functional elucidation of these complexes.

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Conflict of interest

There is no conflict of interest to disclose.

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