Chapter 2

Molecular Insight into Specific 14-3-3 Modulators: Inhibitors and Stabilisers of Protein-Protein Interactions of 14-3-3

The 14-3-3 protein family is implicated in several diseases and biological processes. Several recent reviews have summarised knowledge on certain aspects of 14-3-3 proteins, ranging from a historic overview to the structure, function and regulation. This chapter focuses on the structures and molecular recognition of the modulators by the 14-3-3 proteins, and small modifications of certain modulators are proposed where cocrystal structures have been reported. Our analysis opens up possibilities for the optimisation of the reported compounds. It is very timely to analyse the current status of recently developed modulators given that the field has seen a lot of activity in recent years. This chapter provides an overview combined with a critical analysis of each class of modulators, keeping their suitability for future development in mind.

This chapter has been published as a review article:

2.1 Introduction

Protein-protein interactions (PPIs) play a significant role in many diseases. While a single polypeptide chain can have enzymatic or structural activity, interactions with other proteins allow an infinite variety of ways to modulate the activity. It is assumed that between 130,000 and 650,000 PPIs exist, making PPIs important therapeutic targets. This especially holds for diseases, which are difficult to treat, for example when targets like ion channels, enzymes and GPCRs have not yet been identified or are not present at all.[1]

One class of proteins in which PPIs play an important role is the 14-3-3 protein family. This protein family consists of seven isoforms in humans: beta (β), epsilon (ε), eta (η), gamma (γ), tau (τ), sigma (σ) and zeta (ζ). Each of these human isoforms is encoded by a distinct genetic sequence. While plants have been shown to have more than ten isoforms, eukaryotic micro-organisms have fewer isoforms, for instance, two isoforms have been revealed in yeast.[2] All isoforms have a high conformational and functional conservation. The two isoforms found in yeast can be interchanged with plant or mammalian isoforms and still be active.[2] It has been shown that the binding groove of all isoforms has three conserved binding motifs: RSXpSXP (mode 1), RXXXpSXP (mode 2) and pS/TX-COOH (mode 3), in which pS stands for a phosphoserine residue.[3,4] 14-3-3 proteins primarily form dimers, which both feature a U-shaped binding groove.[5,6] A superimposition of the seven isoforms is shown in Figure 1, illustrating the conserved conformation of the monomers.[7] Wang and Shakes have made multiple alignments of forty-six sequences of plant, animal and fungal species to determine the molecular evolution of the 14-3-3 protein family.[8] Wilkert et al. also show a sequence conformation among the 14-3-3 proteins.[9]

![Figure 1](image.png)

**Figure 1.** Superimposition of monomers of the seven human isoforms.
The 14-3-3 monomers are acidic (pI ≈ 4.6)\textsuperscript{[10]}, have a molecular weight of 25–30 kDa and can combine to form homo- and heterodimers.\textsuperscript{[11]} The non-trivial name 14-3-3 was assigned to this protein family by the researchers who first identified it from a classification study on brain proteins, due to position of the bands on 2D diethylaminoethyl (DEAE)-cellulose chromatography and starch gel electrophoresis.

The 14-3-3 family is implicated in several diseases and biological processes. Studies on binding partners of the 14-3-3 proteins have revealed over 500 proteins, and this number will continue to grow with ongoing research.\textsuperscript{[12]} Among these binding partners are many biologically relevant targets. In some diseases, the 14-3-3 proteins have inhibitory effects, while in other cases they lead to stabilisation. It is even observed that binding of 14-3-3 enhances the activity of the enzyme N-acetyltransferase (AANAT).\textsuperscript{[3]} Examples of such processes are: regulation of metabolism, signal transduction, cell-cycle control, apoptosis, protein trafficking, transcription, stress response and malignant transformation.\textsuperscript{[5]} A few examples of the many diseases in which 14-3-3 proteins are involved are presented in Table 1.

Table 1. Few examples of many diseases in which 14-3-3 proteins are involved.

<table>
<thead>
<tr>
<th>14-3-3 isoform</th>
<th>Disease/ function</th>
<th>Binding partner</th>
</tr>
</thead>
<tbody>
<tr>
<td>(σ) and (ζ)</td>
<td>Noonan Syndrome\textsuperscript{[13]}</td>
<td>C-Raf</td>
</tr>
<tr>
<td>(ζ)</td>
<td>Cell death\textsuperscript{[14,15]}</td>
<td>Pathogenic protein Exoenzyme S</td>
</tr>
<tr>
<td>(σ) specifically</td>
<td>Oncogenesis and cellular response in DNA damage\textsuperscript{[9,16]}</td>
<td>p53</td>
</tr>
<tr>
<td>all</td>
<td>Mutant (breast) cancer\textsuperscript{[17,18]}</td>
<td>Raf</td>
</tr>
<tr>
<td>(σ) and (ζ)</td>
<td>Alzheimer’s\textsuperscript{[19]}</td>
<td>Tau</td>
</tr>
</tbody>
</table>

In order to develop treatments for these diseases, it will be necessary to modulate PPIs of the relevant proteins. An approach to modulate the PPIs is by the use of small-molecule modulators. Modulators of the PPIs of 14-3-3 with partner proteins can come in two types: inhibitors or stabilisers. Small-molecules modulators are usually derived from natural products such as proteins, peptides or secondary metabolites. These modulators have complex structures, making them specific and selective for 14-3-3 proteins.\textsuperscript{[20]} As the isoforms are encoded by distinct genes, they have a unique but similar amino acid sequence. Due to this
uniqueness it might be possible to selectively modulate a single isoform, leaving the other isoforms untouched. Notably, this selectivity is quite essential from a therapeutic point of view, as the drug should not disturb any other biological processes. Research into the discovery and optimisation of small-molecule modulators should therefore take the selectivity of the compounds into consideration, wherever applicable.

Here, we systematically explore the binding of small-molecule modulators of 14-3-3 proteins at the molecular level, that is, the exact binding of the modulators to the amino acid residues lining the binding groove, aiming to provide an insight into the way these modulators work. For almost all modulators in this chapter of which a crystal structure is known we propose modifications based on a structure-based analysis. Therefore, this chapter gathers knowledge, which can be used for structure-based design of (new) modulators. Several recent reviews have summarised knowledge on other aspects of 14-3-3 proteins: the structural basis,[6] insights from genome-wide studies,[21] a historic overview,[22] the structure, function and regulation,[7] an update on 14-3-3 sigma related to human cancer[11] and small-molecule modulators with a focus on the discovery and the biochemical relevance of the compounds.[12]

2.2 Structure-based optimisation
There are cocrystal structures available of almost all 14-3-3 isoforms (some of which are in complex with interaction partners). 14-3-3 can therefore be conveniently targeted using a structure-based approach. The analysis of the binding modes of the modulators described in this chapter gives insight into where the compounds might be extended or modified in order to achieve higher affinity.

For the known cocrystallised modulators, we suggest small modifications, based on a first inspection of the experimentally determined binding mode analysed in silico. More advanced docking studies and biochemical evaluations should be performed to confirm the improved affinities. The computer programme SeeSAR was used for the evaluation, using the in-built scoring function HYDE.[23–25] As explained by Reulecke et al., HYDE focusses on hydration and desolvation, taking into consideration the local hydrophobicity, solvent accessible surface and contact surface area. It calculates this affinity for every atom present in the interface.[25]
2.3 Inhibitors

The first compounds reported to inhibit the PPIs of 14-3-3 with partner proteins were 1 and its dimer difopein (dimeric fourteen-three-three peptide inhibitor) (2) (Figure 2). The former, a 20-amino acid peptide of Mw 2309.6, was identified by Wang et al. from a phage display to 14-3-3(τ). Petosa et al. showed via a crystal structure (Figure 3) that R18 binds in the amphipathic groove and that the central WLDLE motif is hosted in the part of the pocket that binds phosphonic acids, also called the phospho-accepting pocket. Moreover, the peptide does not require phosphorylation to bind to 14-3-3, as the negatively charged Asp and Glu mimic the contact points of phosphoserine. As seen from HYDE scoring in the programme SeeSAR, there are multiple favourable and some unfavourable interactions between 1 and 14-3-3(ξ) (Figure S1 in the supporting information). The C-terminal Leucine of 1 is involved in lipophilic interactions with Gly169 and Ile217. The carboxylic acid terminal group of 1 shows electrostatic interactions with Arg56 and Arg127. Also the oxygen atom of the most left amide bond shows to lose energy due to desolvation.

Compound 1 does not have selectivity between isoforms of 14-3-3. However, it is selective for 14-3-3 proteins. Binding by 1 inhibits binding of partners like Raf-1, ASK1 and exoenzyme S from binding to 14-3-3 in vitro. Fu and Masters reported that 2, enhances the ability of the antineoplastic drug cisplatin to kill cells. Compound 2 can bind competitively to one or two monomers of 14-3-3 given that the two 1 repeats are separated by a flexible linker. Cao et al. showed that 2, as a general antagonist of 14-3-3, effectively hinders proliferation and triggers apoptosis of tumour cells, which were implanted in nude mice. Via flow cytometric analysis it was shown that apoptosis is time-dependent.

The first small-molecule inhibitor of 14-3-3 proteins was compound 3 (Figure 4), which specifically and competitively inhibits 14-3-3/ligand binding in vitro. Wu et al. demonstrated that 3 also causes apoptosis and cell-cycle arrest in mammals. The central motif of 3 is a phosphorylated peptide-like small molecule; its non-phosphorylated analogue shows no activity in competing with 14-3-3(σ)/ligands. Molecular docking was used by the authors to confirm the binding mode of 3 with 14-3-3(σ); no cocrystal structure is available. Optimisation of 3 led to compound 4. This prodrug is a phosphoserine mimetic and potent 14-3-3 inhibitor. When compound 4 is used as a prodrug, the active compound, the phosphonic acid is delivered more effectively into the cells.
Figure 2. Structure of the WLDLE motif which is found in the phospho-accepting pocket of 14-3-3 proteins.[27] And the sequence of 1, highlighted is the WLDLE motif.

Figure 3. Cocrystal structure of 14-3-3(ζ) with 1 (PDB code: 1A38). Colour code: protein surface: grey; interacting residues: sticks; inhibitor skeleton: C: yellow; N: blue; O: red, Cl: green; H-bonds, below 3.2 Å: green, dashed lines. This colour code is maintained throughout this chapter.[28]

Figure 4. Inhibitors 3 and prodrug 4, identified via an small-molecule microarray (SMM) technique consisting of fragment-based high-throughput identification combined with a reconstitution step and biological validation.[30]
Almost concurrently with the discovery of 4, Corradi and et al. disclosed the non-peptidic small-molecule 5, an inhibitor of the 14-3-3/c-Abl complex (Figure 5). The authors used an in silico virtual screening campaign and docking simulations, based on a structure-based pharmacophore model, against 200,000 compounds. Compound 5 can be used to treat Bcr-Abl-associated diseases, which are resistant to traditional drugs.[32] Unfortunately, it was found that 5 is chemically unstable, as it cyclises at room temperature.[33] The authors carried out a virtual-screening campaign with the aim of identifying compounds that score better than 5. Compounds 6 and 7 emerged as hits that are cytotoxic against Bcr-Abl-expressing Ba/F3 cells, and according to molecular modelling bind to 14-3-3(ζ) in a manner similar to that of 5 and 1.[33]

![Chemical structures of inhibitors 5, 6, and 7](image)

**Figure 5.** Inhibitors 5, 6 and 7, identified via a virtual screening campaign.[32]

Another small-molecule inhibitor of all 14-3-3 isoforms in 14-3-3 PPIs, FOBISIN101 (8), was reported in 2011 (Figure 6).[34] FOBISIN (FOurtien-three-three BInding Small molecule INhibitor) 101 is a phosphoSer/Thr-mimetic agent, which upon X-ray irradiation covalently binds to 14-3-3(ζ) at Lys120. Furthermore, it has lipophilic interactions with Pro167, Gly171 and Ile219 and polar interactions to the flexible Lys49. Zhao et al. observed a colour change from bright orange before X-ray irradiation to brownish then yellowish and finally colourless after irradiation. In addition to this covalent linkage, which plays a role in its inhibitory activity, the authors suggest that the phosphate moiety might play a critical role. This conclusion was drawn from the observation that a non-phosphorylated derivative of 8 is significantly less effective in blocking 14-3-3/ligand binding. Mutation of Lys120 to Glu was shown to inactivate 14-3-3(ζ), this was observed in an assay to determine the activation of ExoS ADP-ribosyltransferase. Therefore, this indicates that Lys120 plays an important role in the binding to ligands/modulators.[34] The authors noted that another covalent linkage might also occur through imine formation, which was proposed later by Röglin et al. and is shown in Figure 7. Röglin et al. propose a likely mechanism
involving imine hydrolysis to a pyridoxal-phosphate (PLP) intermediate followed by imine formation with Lys122 of 14-3-3(ζ). The differences in experimental conditions appear to determine whether the covalent linkage takes place. With the Cu-Kα edge radiation they did not observe a colour change/covalent linkage as previously described. However, synchrotron irradiation did result in the covalent modification at Lys120 as described before. They agree that the risk of nonspecific imine formation might be prevented by eliminating the aldehyde functionality, thereby making it a more potent class of compounds.

Figure 6. Inhibitors FOBISIN101 (8) and the covalent adduct 9 with 14-3-3(ζ). Compound 8 was identified from a fluorescence polarisation-based 14-3-3 binding assay, screening compounds that disrupt the 14-3-3(γ)-pS259-Raf-1 peptide complex.

Figure 7. Cocrystal structure of 9: compound 8 covalently linked to Lys122 of 14-3-3(σ) via an imine bond (PDB code: 3U9X).
Shortly after the publication on 8, Park et al. reported on xanthine (10) and lacosamide (11) derivatives as specific binders of 14-3-3(ζ) (Figure 8). Using in vitro labelling, they showed that the enantiomer R-11 binds specifically to 14-3-3(ζ). The stereoisomer S-11 was found to be less active, and R-12 outcompetes R-11 for binding to 14-3-3(ζ). The authors were unable to explain the isoform selectivity, cocrystallographic data could help to gain a better understanding.[37]

![Xanthine (10)](image1)

(R-11) and (S-11)

(R-12)

**Figure 8.** Modulator Xanthine (10) and inhibitors lacosamide (R) and (S)-11 and derivative (R)-12. 14-3-3 was identified as a binding partner to these compounds from a ligand affinity and chemical reporter strategy.[37]

A high-throughput screen using an in vitro fluorescence-polarisation assay afforded another class of scaffolds that modulate 14-3-3/ligand interactions, namely compounds with the core structure 13 (Figure 9).[38] The main part of the PRLX-scaffold is a 1,2,4-thiadiazole, decorated with substituents (R), which were not specified as they were patented compounds. There is no x-ray crystal structure published with these modulators. The authors used multiple biophysical assays to assess the activity of the compounds, starting with isolated cells and followed by intact tissue. Five different 14-3-3 isoforms from E. coli ((β, ζ, η, ε and γ) were tested and it was found that the compounds had highest binding affinity for the (γ) isoform.[38] The origin of this specificity was left uninvestigated. The thiadiazole scaffold of compound 13 (Figure 4) inhibits the interaction of 14-3-3(γ) and a phosphorylated HSP20 peptide (6-FAM-WLRRApSAP).

![13](image2)

**Figure 9.** Inhibitor scaffold 13 identified by high-throughput screening.[38]
In 2011, the inhibitor 14 was identified in a study aimed at making derivatives of the natural product class of the moverastins, which target cell migration of tumour cells for a possible treatment of tumour metastasis. Compound 14 (Figure 10) was found to interact differently than moverastins, therefore suggesting a different target than the original target of the study, farnesyltransferase (FTase).\textsuperscript{[39]} A follow-up study confirmed 14-3-3(ζ) as the target, and from the seven human isoforms it was found that (ζ) had the strongest binding affinity. The authors suggest that this selectivity stems from the fact that the C-terminal region of 14-3-3 is involved in the binding, which is the most variable region of the isoforms. Compound 14 presumably inhibits cell migration by competing with the 14-3-3(ζ)/Tiam1 complex.\textsuperscript{[40]}

![Figure 10. Inhibitor 14, a moverastin derivative that targets 14-3-3(ζ).\textsuperscript{[39]}](#)

In 2012, Yan et al. identified Blapsins A (15) and B (16) as potent small-molecule inhibitors. These compounds (Figure 11) were isolated from Blaps japonensis, characterised and evaluated for their inhibitory potencies on 14-3-3(γ).\textsuperscript{[41]} They were found to inhibit 14-3-3 binding to the fluorescence-labelled TMR-Raf pSer259 peptide, used for monitoring protein interactions.\textsuperscript{[42]}

![Figure 11. Inhibitors Blapsin A (15) and Blapsin B (16), isolated from Blaps japonensis.\textsuperscript{[41]}](#)

A virtual-screening campaign and subsequent experimental validation revealed compound 17 (Figure 12) as a potent inhibitor of 14-3-3(ζ).\textsuperscript{[43]} Compound 17 has a structure similar to that of 15, except that 17 has a phosphonic acid, which serves as an anchor in the binding pocket of 14-3-3. The authors screened the ZINC all now subset in order to find a non-peptidic and non-covalently binding small-molecule inhibitor of the extracellular PPI of 14-3-3 with the membrane receptor aminopeptidase N (APN). In addition to disruption of the 14-3-3(σ)/APN complex, compound 17 was also found to inhibit the overexpression of
matrix metalloproteinase (MMP)-1 in human fibroblasts, which was induced by signalling of 14-3-3 proteins. The binding mode of 17 to 14-3-3 proteins was validated by solving a cocrystal structure in complex with 14-3-3(σ) (Figure 13).[^43]

Compound 17 binds strongly due to the phosphonic acid. Analysis with the programme SeeSAR revealed that both chloride atoms are involved in lipophilic interactions with Arg60, Arg56 and Ala57, the oxygen atoms at the phosphonic acid anchor are involved in strong H-bonding interactions with Arg129 and Arg56. The carbon atom para to the phosphonic acid does not contribute to affinity. This problem might be overcome by installing a hydroxyl moiety on that carbon, as was modelled in the SeeSAR programme (see the supporting information Figure S2 and S3).

![Figure 12. Structure of virtual-screening hit 17, a potent 14-3-3 inhibitor.][^43]

![Figure 13. Crystal structure of virtual-screening hit 17 in complex with 14-3-3(σ) (PDB code: 4DHU).][^43]

In 2014, Glas and coworkers reported that constrained peptides can serve as inhibitors of pathogenic PPIs. They used target-adapted cross-links to stabilise secondary structures in order to inhibit the interaction of 14-3-3 with virulence vector exoenzyme S (ExoS).[^15] The ESp sequence was used as a starting point for the design of macrocyclic structures. This ESp sequence, 420QGLLDALDLAS430, had previously been identified as a binding motif.[^14] The authors compared the binding mode of their cross-linked peptide, 18 (Figure 14), to that of ESp by
solving the crystal structure of both ligands with 14-3-3(ζ) (Figure 15 and supporting information Figure S4). Both ligands were shown to lie in the same binding site, 18, however, has a 20-fold higher binding affinity. The authors suggest, via NMR, CD and ITC experiments, that the improved binding entropy for the cross-linked peptide is due to the reduction of conformational flexibility.\[15\]

![Figure 14](image1.png)

**Figure 14.** Cocrystal structure of cross-linked peptide 18 with 14-3-3(ζ) (PDB code: 4N84).\[15\]

![Figure 15](image2.png)

**Figure 15.** Structure of the constrained peptide 18, an inhibitor of 14-3-3(ζ).\[15\]

A novel modulator of 14-3-3/ligand complexes was introduced by Milroy et al.. They started with the stabiliser fusicoccin A (FC) (19) (Figure 17) in order to
design inhibitors, since they found that the region on 14-3-3 where 19 binds partially overlaps with the region where known inhibitors bind. The part of the active site from which stabilisation is observed lies next to the part where inhibition is observed, that is, there is a small interface between the modulating effects of these parts. The Tau pSer214 epitope was modified with different amines to obtain higher affinity. Milroy et al. hypothesised that using the OMe substituent of 19 would give high affinities due to interactions with the FC pocket, consisting of Phe, Met and Lys residues.\textsuperscript{[19]}

The Tau epitope, AcNH-RTPpSLPTP-OH, decorated with compound 20 (Figure 16) showed to have good affinities for 14-3-3 due to the OMe group. Yet, tau epitope expanded with 21 emerged as the most active inhibitor, the bulky aromatic groups increased the affinity. This compound inhibits the binding of phosphorylated full-length Tau (protein) to 14-3-3. It has a 225-fold higher affinity than the Tau epitope. Inhibitors of 14-3-3/Tau might be useful in the treatment of Alzheimer’s disease. However, the cocrystal structure they reported is in complex with amine 20, which has a fifteen-fold worse $IC_{50}$ value than 21 (see the supporting information Figure S5).\textsuperscript{[19]}

![Figure 16. Cocrystal structure of 14-3-3(σ) and phosphorylated Tau epitope with amine 20, identified using a structure based drug design (SBDD) approach (PDB code: 4Y3B).\textsuperscript{[19]}](image)
2.4 Stabilisers

The natural product fusicoccin A (19) was reported as the first stabiliser of PPIs of 14-3-3/ligands. This diterpene glycoside, member of a class of diterpenes bearing a 5-8-5 ring structure called fusicoccanes, was found to bind to 14-3-3 receptors.\[44\] 19 molecularly glues together the 14-3-3/ plasma membrane ATPase complex (PMA), while it does not bind to the individual partners.\[45\] The PMA is bound at the C-terminus via a phosphorylated Thr. The scaffold of 19 provides a starting point for the design of further stabilisers of 14-3-3/ligand complexes, as it interacts on multiple sites with both protein and ligands.

19 was found to not only stabilise the plant plasma membrane pump but also the binding of 14-3-3 to estrogen receptor alpha (ERα). The ERα is an anticancer target given that stabilising the 14-3-3/ERα complex has a downstream effect and leads to decreased cell proliferation.\[18\]

Binding of 19 to PMA2 can probably be enhanced by changing the oxygen on the oxane ring for a carbon atom (Figure 17 and supporting information Figures S6–S8). In this case, the programme SeeSAR predicts a better affinity, and Hyde evaluation only reveals favourable interactions. A similar improvement can be achieved in 19 with the 14-3-3/ cystic fibrosis transmembrane conductance regulator (CFTR) complex: the oxygen atom bridging the two rings is found to partially disrupt binding. The binding is predicted to improve when the oxygen is replaced by a carbon atom (Figure 18 and supporting information Figures S9 – S11).

Figure 17. Crystal structure of the stabiliser fusicoccin (19)-plasma membrane ATPase complex 2-C52 in the tobacco 14-3-3c isoform (PDB code: 2O98).\[46\]
Using 19 as a starting point, Anders et al. have made a semisynthetic derivative (22) (Figure 19). This derivative targets 14-3-3 in the human potassium channel TASK-3 and interacts in the mode 3 motif of the conserved binding groove. 22 leads to a 19-fold increased binding of 14-3-3 to TASK-3.[48]
interactions and a better affinity than 22 (see supporting information Figures S12 – S14).

Cotylenin A (CN-A) (23) is a natural product closely related to 19. Compound 23 has anti-tumour activity by stabilising certain PPIs involved in human cancers. It is assumed that the hydroxyl moiety on the cyclopentenyl ring in 19 might determine the structural specificity compared to 23 and related fusicoccanes.[49] Four years after the work of Ottmann, Molzan et al. reported that the 14-3-3/C-Raf complex is stabilised by 23 (Figure 20). Cotylenin A interacts with pSer233 and pSer259 of C-Raf. At these positions, 14-3-3 binding inhibits C-Raf, whereas at pSer621, 14-3-3 binding activates C-Raf.[50,51] The in vivo activity of 23 on 14-3-3/C-Raf is increased compared to the in vitro activity. This difference indicates that other processes are also influenced by 23.[17]

Figure 20. Cocrystal structure of stabiliser cotylenin A (23) and C-Raf in 14-3-3(ζ) (PDB code: 4IHL).[17]

Compound 23 displays an unfavourable interaction at the oxygen atom bridging the two ring structures. Substituting this oxygen for a nitrogen atom could increase the affinity by having an additional H-bond towards Asp-213 (Figures S15 – S17 in the supporting information). The two six-membered rings are now less involved in binding, however changing the bridging oxygen atom for a nitrogen atom should lead to an increased affinity.

In 2010, Rose et al. identified small-molecule stabilisers of 14-3-3 PPIs, namely a tri-substituted pyrrolidone (24), and a dipeptide epibestatin (25). The authors screened a 37000-member compound library using green fluorescent protein
(GFP) fused with 14-3-3 against binding to surface-immobilised glutathione S-transferase fused with PMA2-CT52. The selective stabilisation of 14-3-3/PMA2 by 25 displays a slower association than 19 and 24, but a similar $K_d$ to 19. In other words, stabilising the PPI with 25 is relatively slow but once it has been established it will bind strongly (Figure 21). It was found that compound 26 has a stabilising effect on 14-3-3/PMA2 that is three times stronger than that of 24 (Figures 21 and 22), presumably because more contact points with the PMA2 residues are established.

![Figure 21. Crystal structure of stabiliser epibestatin (25)-plasma membrane ATPase complex 2-CT30 in 14-3-3 (PDB code: 3M50). 25 was identified by screening a compound library against binding of 14-3-3 to plasma membrane ATPase complex 2-CT52.](image)

The programme SeeSAR was used to suggest some modifications. Substituting the free amine in epibestatin (25) by a carbon atom, for instance, enhances the binding to the PMA moiety. However, this still gives clashes; changing it into a hydroxyl group makes it a bit smaller and again an H-bond acceptor/donor. With an alcohol moiety the affinity most probably increases again, but clashes are still seen with Leu929 and His930 (see Figures S18 – S20 in the supporting information).

The hydroxyl group of the central five-membered ring in 24 appears to have some unfavourable interactions with Val45 and Asn49. Replacing the hydroxyl group for an amine improves the affinity a bit, going to fluoride improves the binding even more (Figures S21 – S23 in the supporting information).
Mizoribine (27), or bredinin (Figure 24), is a compound isolated from *Eupenicillium brefeldianum* and found to have activities in multiple animal studies as well as an immunosuppressive activity.[54] This imidazole nucleoside enhances the interaction of glucocorticoid receptor (GR) with 14-3-3(η) *in vitro*.[55] Mizoribine has been an approved drug in Japan for many years for the treatment of lupus nephritis, rheumatoid arthritis and for immunosuppressive therapy after renal transplantation.[55]
The most recently published small-molecule stabiliser 28 holds promise to be used in new therapeutic approaches to target Gab2-related cancers. Gab2 is a signalling protein,[56] which is correlated to the mitogen-activated protein kinase (MAPK) pathways involved in certain cancer types (e.g., breast cancer).[57] Bier et al. showed in an elaborate study that the FC-derivative 28 stabilises the binding of pT391 peptides, which are derived from the binding moiety of Gab2 (Figure 25). The stabilisation is specific for the pT391 motif, as no enhanced binding was observed for the other phosphorylation site of Gab2, the Gab2pS210 binding motif. The authors examined the binding interactions of Gab2pT391 and Gab2pS210 via cocrystal structures.[58] It was shown that, in the pT391 motif, Leu392 of Gab2 can be best accommodated in the hydrophobic contact surface of Leu172, Ile217 and Leu220 of 14-3-3(ζ). Gab2 motifs are anchored through the phosphonic acid in the binding triad of Arg56, Arg127 and Tyr128, as is frequently observed and described in this chapter. The cocrystal structure of 14-3-3(ζ) with Gab2 and 28 is given in Figure 26. The hydrophobic pocket in 14-3-3(ζ), consisting of the following amino acids Val46, Phe117, Met121, Pro165, Ile166, Gly169 and Ile217, is accommodated by the terpenoid core of 28 and by Leu392 of Gab2. The Gab2pT391 peptide has a sharp turn, most probably induced by Pro393, which therefore gives space for 28 to bind and stabilise the complex. This turn is not observed in Gab2pS210, which leads to the postulation by the authors that the difference of one or two amino acids in the 14-3-3 recognition motifs of Gab2 could be used to design PPI-target-specific stabilisers.[58]

Figure 25. Stabiliser 28, which stabilises the complex of Gab2pT391 peptide and 14-3-3(ζ).[58]
The programme SeeSar was used to analyse the binding affinity of 28 to Gab2pT391 peptide and 14-3-3(ζ). The methoxy carbon atom at the terpenoid core has strong interactions with Met121 and Phe117. The remaining carbon atoms are also involved in hydrophobic interactions with the protein. For example, the isopropyl moiety has strong interactions with Asp213, Ile217 and Leu392. However, the bridging oxygen atom towards the two six-membered rings shows to be non-favourable. It forms an intramolecular H-bond, but it loses affinity due to desolvation. Substituting this oxygen atom for a carbon atom might improve the binding affinity, as this carbon atom could then have lipophilic interactions with Asp213. The terpenoid core should remain in the hydrophobic part, and the two six-membered rings which are solvent exposed move slightly. (Figures S24 – S26 in the supporting information).

2.5 Development in the discovery of modulators of 14-3-3 proteins since 2016

Eighteen years ago Camoni et al. have shown that Adenosine 5’-Monophosphate (AMP) inhibits 14-3-3 proteins binding to the plant plasma membrane H^+-ATPase (PMA). At a concentration of 1 mM, 5’-AMP inhibits binding of 14-3-3 to a peptidic analogue of the binding site of the H^+-ATPase. However, it has been shown in 2016 that AMP can also act as a stabiliser. This small molecule was found to stabilise the complex of 14-3-3 with carbohydrate-response element-binding protein (ChREBP). 5’-AMP binds the complex with a $K_d$ of 7.6 ± 0.7 µM,
where under the same conditions no binding was observed to the complex partners alone.\[61\]

Cyclotides, plant-derived circular peptides, are a promising class of molecules in drug discovery and development. Hellinger et al. have used photo-affinity crosslinking of the circular peptide [T20K]kalata B1 (Table 2) and found that it targets 14-3-3 proteins. It was found that this cyclotide exerts a stabilizing effect on the 14-3-3/Foxo3a protein complex. By stabilizing the complex, [T20K]kB1 inhibited the function of Foxo3a with an IC$_{50}$ of 5.3 ± 0.7 µM. A similar way of stabilizing 14-3-3 complexes as is known for fusicoccin-A, was observed for the cyclotide.\[62\]

**Table 2.** Cyclotide probe, found to modulate 14-3-3 proteins.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Molecular weight (Da)</th>
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</table>

In a joint effort, teams of the University of Eindhoven, University of Duisburg-Essen and AstraZeneca reported on the first small-molecule stabiliser of the 14-3-3–p53 interaction in 2017. They showed that fusicoccin-A 19 stabilises the PPI of 14-3-3 with p53, rather than the previously hypothesised opposite. Biophysical experiments show a clear stabilising effect, however the cocrystal structure showed more disorder. The authors hypothesise that this could be due to artefacts of soaking the crystal or that 19 acts allosterically instead of the expected mode-of-action as a `molecular glue`. The binding of p53 C-terminal domain 15mer peptide to 14-3-3 was found to be stabilized by 19 by a factor of 4.5 – 8 (K$_d$ 23.6 ± 2.2 µM in absence of 19, and 5.40 ± 0.84 µM in presence).\[63\]

In 2018, Andrei et al. have reported on the rational design of new semisynthetic analogues of fusicoccin-A to stabilise PPIs (Figure 27). They included a hydrogen-bond donor by exchanging the 19-acetoxy group for an acetamide, moreover they removed the 3′-acetyl group which did not significantly contributed to the potency. The stabilisation factor S was increased by 29 and 30 up to 16-fold for different 14-3-3 complexes, compared to 19. In a cell growth assay, using a human ovarian carcinoma cell line, EC$_{50}$ values of 4.3 ± 0.3 µM for 29 and 2.5 ± 0.1 µM for 30 were found.\[64\]
Figure 27. Semisynthetic analogues of fusococin-A (19) via Structure-based design and molecular dynamics.[64]

In a different study by this author, it was demonstrated that full length Tau protein could be inhibited from binding to 14-3-3 by synthetic peptide analogues of Tau. These compounds bind 14-3-3 with $K_d$ values in the range of 2.7—20.6 µM.[65]

The groups of Schmuck and Ottmann have recently put forth a new class of supramolecular peptide-based ligands which stabilise 14-3-3ζ and two effectors, Tau and C-Raf. The binding by positively charged compounds is designed around the anionic region in the central binding channel of 14-3-3 (Figure 28). They implemented a guanidinocarbonyl pyrrole (GCP) moiety, an arginine mimic, to form stable ion pairs with carboxylates and phosphates. Compound 31 was the most potent derivative, enhancing the interaction of 14-3-3ζ to C-Raf and Tau with $S$ factors of 84 and 26. Binding partner C-Raf’s $K_d$ value improved from $16.00 \pm 0.60 \mu M$ to $0.19 \pm 0.01 \mu M$ and binding partner Tau’s $K_d$ value improved from $7.00 \pm 0.20 \mu M$ to $0.27 \pm 0.01 \mu M$.[66]

Figure 28. Supramolecular peptide-based ligands, 31, 32 and 33, stabilising 14-3-3ζ and two effectors, Tau and C-Raf. Binding was mainly due to the formation of ion pairs through a guanidinocarbonyl pyrrole moiety with carboxylates and phosphates.[66]
Another recent publication was inspired by 5 (BV02) in order to find chemically more stable compounds. It was found that the bioactive form of 5 is the phtalimide 34, which is formed via a hydration/dehydration pathway (Figure 29). Botta and coworkers have removed a carbonyl group of 34, which was found to take place in the hydrolysis and made the chemically more stable compound 35. It was found, in an in vitro antiproliferative activity study, that 35 has an \( IC_{50} \) value of 7.7 ± 2.0 \( \mu \)M, similar to the \( IC_{50} \) value of 5.2 ± 0.7 \( \mu \)M for 34.[67]

\[ \text{Figure 29.} \text{ Bioactive form of 5 was shown to be the phtalimide 34. Botta and coworkers synthesised 35 in order to increase the chemical stability of the small-molecule inhibitor of 14-3-3.}[67] \]

The interaction of 14-3-3 with a phosphorylated motif derived from Estrogen Receptor α (ERα-pp) was used as a PPI complex in a site-directed fragment-based screening campaign. Based on disulfide trapping of fragments, Ottmann and coworkers have found orthosteric stabilisers, increasing the 14-3-3/ERα affinity up to 40-fold (Figure 30). The apparent \( K_d \) value of 14-3-30/ERα-pp improved from 1.3 \( \mu \)M to 32 nM in presence of 36.[68]

\[ \text{Figure 30.} \text{ Small-molecule 36, found via disulfide trapping with 14-3-3, stabilising the complex of 14-3-3/ERα.}[68] \]
2.6 Conclusions
Research has focussed, for almost 20 years now, on the development of 14-3-3 inhibitors and stabilisers. We have analysed the structures of the reported inhibitors and stabilisers. Visual inspection of the cocrystal structures of the published small-molecule modulators at the molecular level aided by the programme SeeSAR has led to the proposal of small modifications, which might improve the binding affinity. The knowledge gained from exploring the cocrystal structures sets the stage for structure-based design of (new) modulators. Designing molecules to stabilise or even activate 14-3-3/partner protein complexes has the potential to become an important part of modulating 14-3-3 PPIs, since research has focussed so far more on inhibiting PPIs.

Since there are seven isoforms, with a high degree of conservation, it might be wiser to target the interaction partner rather than 14-3-3. This approach might lead to more specific small-molecule modulators that target a specific PPI instead of all PPIs from the 14-3-3 protein. This would require a paradigm shift, as research has focussed on binding to 14-3-3. Focussing on the interaction partner might give more freedom and creativity when aiming to influence PPIs.

2.7 References


[59] BioSolveIT GmbH, SeeSAR 5.5.


