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Structure, function, involvement in diseases and targeting of 14-3-3 proteins: an update

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Abstract: 14-3-3 is a class of proteins able to interact with a multitude of targets by establish protein-protein interactions (PPIs). They are usually found in all eukaryotes with a conserved secondary structure and high sequence homology among species. 14-3-3 proteins are involved in many healthy and pathological cellular processes either by triggering or interfering with the activity of specific protein partners. In the last years, the scientific community has collected many evidences on the role played by seven human 14-3-3 isoforms in cancer or neurodegenerative diseases. Indeed, these proteins regulate the molecular mechanisms associated to these diseases by interacting with (i) oncogenic and (ii) pro-apoptotic proteins and (iii) with proteins involved in Parkinson and Alzheimer diseases. The discovery of small molecule modulators of 14-3-3 PPIs could facilitate the full understanding of the physiological role of these proteins, and might offer valuable therapeutic approaches for these critical pathological states.

Keywords: 14-3-3, cancer, neurodegeneration, protein-protein interaction, small molecules, drug discovery

1. INTRODUCTION

14-3-3 are phospho-serine/phospho-threonine binding proteins able to associate with a wide range of protein targets, like kinases, phosphatases, transmembrane receptors and transcription factors [1-3]. They are ubiquitously expressed in all eukaryotic organisms and, by interacting with a multitude of functionally diverse and generally phosphorylated molecules, regulate a huge number of physiological processes, such as intracellular protein trafficking, cell proliferation, growth and apoptosis, regulation of metabolism, signal transduction and stress responses [4-7].

Besides, there are many evidences that misregulation of 14-3-3 proteins contributes to important human diseases such as cancer, neurodegenerative disorders, and infection by Giardia intestinalis [8-11]. The development of new and effective treatments for these diseases is one of the main challenges of the scientific community. Although cancer and neurodegeneration are generally considered as two opposite disorders [12-14], it is recently emerging the idea that they might share common etiologic mechanisms and therapeutic targets [15-19] .

In this respect, the evidence that 14-3-3 proteins are down- or up-regulated in both cancer and neurodegeneration suggests that targeting 14-3-3 proteins with drug compounds may open a common therapeutic approach against both diseases.

The aim of this contribution is to review 14-3-3 proteins by focusing on (i) structural features, (ii) interactions with proteins involved in cancer and neurodegenerative diseases and finally (iii) modulators of 14-3-3 protein-protein interactions (PPIs) discovered so far.

2. STRUCTURAL FEATURES AND FUNCTIONS OF 14-3-3 PROTEINS

14-3-3 is a family of about 30 kDa proteins expressed in all eukaryotic cells and composed of different isoforms, whose number differs among species. Seven isoforms were found in humans, encoded by distinct genes and labeled with the Greek letters α/β, γ, ε, ζ, η, σ and θ/τ [1, 20].

All isoforms have a high sequence homology (about 44%) and well conserved structure [21-25]. 14-3-3 proteins exist as dimers and, either homo- or heterodimers are formed by the interaction between the N-terminal regions of identical or different isoforms [26-28]. Each monomeric unit contains nine antiparallel α-helix. In the dimeric structure, helix αl from one monomer tie together helix α3 and helix α4 from its partner by forming a large groove (Figure 1). The association between dimer interfaces is mainly driven by hydrophobic and electrostatic forces between amino acid sidechains belonging to Leu, Ala, Ile, and Tyr. Moreover, the formation of a salt bridge between glutamate and arginine sidechains is highly conserved as well [23, 27, 29].

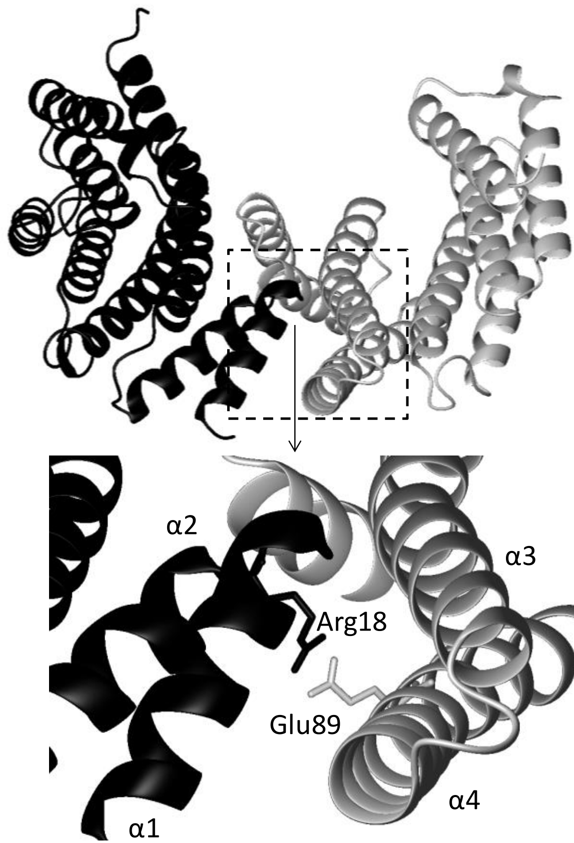

Figure 1. Ribbon representation of the crystal structure of the 14-3-3 human ζ isoform (PDB ID code 1QJB [23]). The two monomers are shown in different colors. Details of dimer interface are shown as expansion of the rectangular frame.

All 14-3-3 isoforms can recognize three high-affinity binding motifs containing phosphoserine (pSer): RSXpSXP (mode 1), RXXXpSXP (mode2) and pS/TxCOOH (mode 3) [23, 30, 31]. These sequences are not the only regions recognized by 14-3-3 proteins, which are also able to interact with different protein sequences containing either phosphorylated or unphosphorylated serine or threonine [22, 32, 33].

14-3-3 proteins are involved in a multitude of biological pathways through different mechanisms. Unfortunately most of the structural data obtained so far, are limited to 14-3-3 proteins associations with short phosphopeptides, thus limiting the understanding of the exact mechanism of protein-protein associations and binding. However, according to their functional roles, three different modes of action have been proposed for 14-3-3 PPIs (Figure 2) [1, 20, 29, 34]. The first one consists on inducing a conformational change of the target protein, which can thus accommodate on the large groove of the 14-3-3 dimeric structure (Figure 2A). Additionally, 14-3-3 proteins can interfere with protein-protein or protein-DNA interactions by occluding specific regions of the target proteins (Figure 2B). Finally, 14-3-3 proteins may favor the association between two other proteins (Figure 2C), which can easily interact once they are hold in the 14-3-3 dimeric groove.

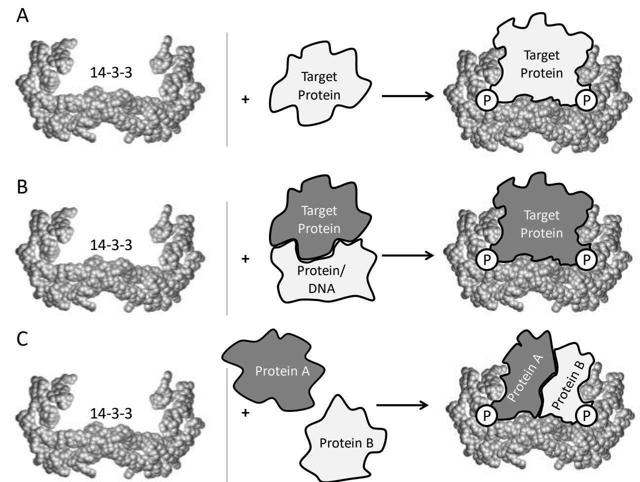

Figure 2. Proposed mechanisms of action of 14-3-3 proteins. Adapted from ref [29].

3. INVOLVEMENT OF 14-3-3 PROTEINS IN HUMAN DISEASES

3.1. Cancer

Several studies have demonstrated the ability of 14-3-3 to target oncogenic proteins, thus suggesting a strong correlation between the aberrant expression of 14-3-3 proteins and many types of cancer [2, 35, 36], even though this correlation is highly sensible to the isoform and the tissue of expression. 14-3-3ζ is associated with a variety of cancers including breast, prostate, lung, ovarian and gastric cancers. High levels of 14-3-3ζ are found in cancer cells and in samples of patient affected by breast cancer and they correlate with poor cancer prognosis [37]. In particular, cancer cell survival and Akt activation is enhanced by 14-3-3ζ interaction with the p85 regulatory subunit of PI3 kinase [38]. Additional markers of breast cancer are low level of 14-3-3γ and θ isoforms [39]. On the other hand, gastric cancer is associated with elevated levels of 14-3-3β [40]. A completely different behavior is observed for 14-3-3σ isoform, which exert both pro-oncogenic and tumor suppressor functions [41]. In the case of breast cancer, there are evidences that correlate the low expression of 14-3-3σ to hypermethylation of the 14-3-3σ promoter, leading to gene silencing [42, 43]. All these data strongly point out the possible use of 14-3-3σ methylation as a diagnostic marker for breast cancer.

In contrast, 14-3-3σ isoform has been found to interact with several pro-apoptotic proteins interfering with their sub-cellular redistribution. In Chronic Myeloid Leukemia, CML, overexpressed 14-3-3σ is able to interact with c-Abl protein inducing its cytoplasmic localization [44]. c-Abl is a non-receptor tyrosine kinases implicated in many cellular processes and its nuclear import is prevented by 14-3-3σ

binding. The domain of 14-3-3σ implicated in c-Abl binding consists in residues 111-155, on the other hand three different regions of c-Abl, encompassing residues 185–209, 548–572 and 729–753 contribute to the interaction with 14-3-3σ.

In addition to c-Abl, 14-3-3σ associates to other proteins linked to cancer etiology, like Raf1, Bad, p53, Cdc25, FOXO and HDAC [35]. This strong correlation between 14-3-3σ and different proteins involved in cancer pathogenesis and progression has lead the scientific community to put a lot of efforts in the discovery of small molecules able to target the 14-3-3σ and to inhibit its PPIs, to ultimately be developed as effective anticancer agents [45-48].

*3.2 Neurodegeneration*14-3-3 proteins are highly expressed in the brain where they constitute about 1% of the total soluble protein content [49, 50]. They are involved in several important processes such as (i) the regulation of neuronal transmission and plasticity at the synapses [51], (ii) neuronal differentiation, migration and survival [8], (iii) ion channel regulation [52] and (iv) neurite outgrowth [8]. It is well established that 14-3-3 proteins also play a relevant role in neurodegenerative diseases, like, Alzheimer's disease (AD), Parkinson disease (PD) and transmissible spongiform encephalopathies (TSEs), among which Creutzfeldt-Jakob disease (CJD) is the most common one [53].

Neurodegenerative diseases are a group of fatal disorders sharing common features and molecular patterns [54-56]. The major hallmarks of these diseases are the abnormal oligomerization and accumulation of misfolded proteins in the brain leading to selective neuronal death. These brain deposits are usually known as Lewy Bodies (LBs) in PD, amyloid plaques and neurofibrillary tangles (NFTs) in AD and prion amyloid plaques in TSEs. The loss of neuronal structure and function is initially restricted to different brain area, such as hippocampus and its connected structures in AD, substantia nigra in PD and cerebral cortex in CJD. These differences results in diverse symptoms: impaired memory and speaking in AD, tremor, bradykinesia and rigidity in PD impaired, judgment, thinking, and vision in CJD.

One of the most important correlation between neurodegenerative diseases and 14-3-3 proteins is that they are found in LBs [8, 57, 58] and in NFTs of AD brain sections [59, 60]. In addition the 14-3-3η isoform is present in the cerebrospinal fluid (CSF) of patients affected by neurodegenerative diseases [61], suggesting a possible role of 14-3-3 proteins as general biomarkers for these disorders. Beside to 14-3-3η isoform, 14-3-3 β, γ and ε are localized in the CSF of CJD patients [62, 63], such that measurements of 14-3-3 levels in CSF is included in the World Health Organization (WHO) diagnostic procedure for CJD [64].

Several 14-3-3 isoforms are able to interact with specific proteins involved either in PD (Figure 3) or in AD (Figure 4).

The interaction between α-Synuclein (αS), the well-known major component of LBs, and 14-3-3 proteins is supported by several findings:

(i) αS and 14-3-3 proteins are both enriched in the synapses [65]. They have physical and functional similarities and they share over 40% sequence homology [66].

(ii) Four of the seven 14-3-3 isoforms (ε, γ, σ, and ζ) were found in LBs in human PD [67]. Association between 14-3-3 (β and ε isoforms) and αS occurs either in cytosolic or membrane fractions of rat brain homogenate [66].

(iii) αS overexpression in human primary neurons cells yields to the formation of a soluble complex between αS and 14-3-3 proteins [68].

(iv) The 14-3-3η isoform strongly affect the kinetics and the products of αS aggregation in vitro by binding to αS oligomers [69]. Overexpression of 14-3-3η isoform results in reduced αS toxicity effects in cellular models as well [69].

Beside to αS, other proteins relevant for PD are able to interact with 14-3-3 proteins: 14-3-3ζ binds and stimulates the activation of tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of catecholamine [70]. Other evidences support 14-3-3η interaction with parkin, an ubiquitin E3 ligase involved in protein degradation [71]. Among the 14-3-3 family, only 14-3-3η, is able to bind to parkin with nanomolar affinity. Such interaction involves the linker region of parkin (residues 76-258), containing –RKDSPP- sequence. Interestingly, 14-3-3η-parkin association causes the suppression of ubiquitin-ligase activity of parkin, which is instead restored when αS is overexpressed. The missense mutant parkin (K161N), causing autosomal recessive juvenile parkinsonism (ARJP), does not interact with 14-3-3η. Similarly, A30P and A53T αS mutants, responsible of familial PD, are not able to bind 14-3-3η and to activate parkin thus suggesting that reduced parkin activity correlates with ARJP and sporadic PD.

Finally, phosphorylated FOXO3a (forkhead box transcription factor, class O) and LRRK2 (leucine-rich repeat kinase 2) are binding partners of 14-3-3 protein as well [72]. FOXO3a localizes in LBs and Su et al. recently proposed the formation of a complex, including FOXO3a, αS, and 14-3-3 proteins, able to promote cell survival [73].

LRRK2 is a 280 KDa protein having serine/threonine kinase and ROC GTPase domains, whose activities are significantly changed in presence of LRRK2 mutations associated with familial and sporadic forms of PD [74-76].

The 14-3-3 proteins binds to the N-terminus of LRRK2 at the phosphorylated Ser910 and Ser935 sites protecting them from dephosphorylation [77].

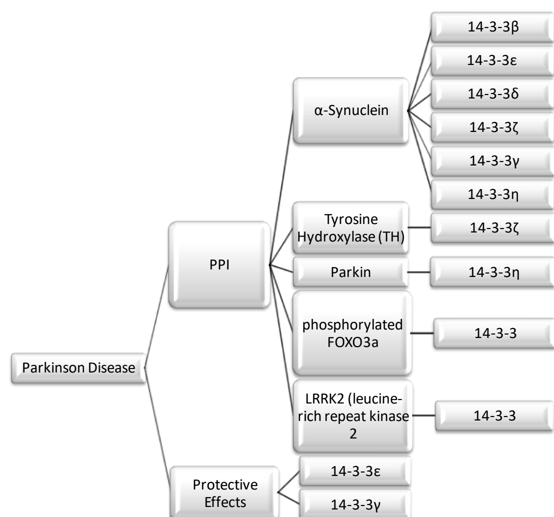

Figure 3. Schematic representation of the involvement of 14-3-3 proteins in Parkinson Disease.

Tau proteins are additional 14-3-3 binding partners. These proteins are highly soluble microtubule-associated proteins which undergo to misfolding and hyper-phosphorylation leading to the formation of NFTs in AD [78]. In particular, GSK3β-mediated phosphorylation of tau is facilitated by the 14-3-3ζ isoform [59, 79-83].

14-3-3ζ also binds to δ-catenin, a brain protein first discovered for the interaction with presenilin 1 [84]. Mutations in δ-catenin are associated with some forms of mental retardation and of early-onset of familial Alzheimer's disease [85-89]. The δ-catenin region –RSApSAP– contains one phosphorylated Ser (Ser1072 in human and Ser1094 in mouse) and is believed to be involved in the interaction with 14-3-3 proteins. In fact Ser/Ala substitutions abolish protein associations [90].

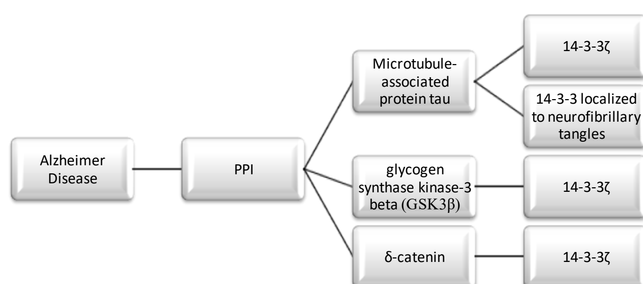

Figure 4. Schematic representation of the interactions between proteins involved in AD and 14-3-3 proteins.

In addition to the evidences of specific interactions with proteins associated to neurodegenerative diseases, 14-3-3 proteins also exhibit protective effects on dopaminergic neurons. Indeed 14-3-3θ, γ and ε isoforms reduce the cellular toxicity induced by neurotoxins causing cell death in dopaminergic cells [91]. It has been also proposed that 14-3-3 proteins are involved in sequestration and degradation of toxic oligomers and aggregates, by favoring aggresome formation [92-94].

4. 14-3-3S AS CANDIDATE DRUG TARGETS

As described above, 14-3-3 have multiple roles depending on the isoform and the site of expression (i.e. the specific organ/tissue/district of the human body). Moreover, by interacting with a wide variety of cellular partners, 14-3-3 proteins are involved in many physiological processes, whereas their aberrant expression has been implicated in a number of human diseases. Small molecules able to modulate the activity of a specific 14-3-3 could be profitable leads for the development of therapeutic agents, as well as valuable tools in chemical biology investigations aimed at further understanding the role of this protein family. To the best of our knowledge, to date there are no 14-3-3 PPIs modulators in preclinical or clinical trials. Similarly, there are no valuable candidates in advanced stages of the development, for which the preclinical development could be undertaken within the next few years. This evidence should point the attention to the need of focusing drug discovery campaigns, most likely sustained by granting institutions of large pharmaceutical companies, as well as the need to identify the ideal candidate endowed with the desired potency, selectivity and physicochemical features.

It is worth mentioning that in the last decade, significant steps forward have been recorded in targeting 14-3-3 by means of small molecules. This is noticeably due to the structural characterization of these proteins by means of X-ray crystallography [21, 24, 29, 31], which paved the way to structure-based drug design boosted by *in silico* or *in vitro* studies [46, 47, 95-99]. Just as example, from our previous review article focusing on structural and functional features of 14-3-3 [21], the number of 3D structures of 14-3-3 in the Protein Data Bank (www.rcsb.org) has more than doubled (currently there are more than 100 records containing 14-3-3 in the title – July 2016) [100]. Besides human and plant 14-3-3s, which are undoubtedly the most investigated, the structure of 14-3-3 expressed in bacteria [25, 101], and protozoa [102-104], has been characterized. In some cases, these structures have been further exploited in drug design exercises [103].

At present, two main strategies have been undertaken to modulate 14-3-3 proteins at molecular level: i) inhibition of 14-3-3 PPIs by small molecules able to bind the 14-3-3's amphipathic groove and to prevent its interaction with physiological partners; ii) stabilization of 14-3-3 PPIs by small molecules able to bind and stabilize the 14-3-3/partner protein interaction [21, 105, 106]. Here, we review the most recent and relevant modulators of 14-3-3 PPIs, which will be

classified into the two main categories according to their mechanism of action. Where available, key structural details of these molecules will be discussed as well.

4.1 14-3-3 PPIs Inhibitors

From a medicinal chemistry standpoint, the inhibition of 14-3-3 PPIs by small molecules represents the most intuitive and canonical strategy. Indeed, starting from the multiple evidences linking 14-3-3 overexpression to a number of human diseases, specific 14-3-3 PPIs inhibitors are thought to be beneficial in many pathological states [45, 46, 107, 108]. A wide variety of 14-3-3 PPIs inhibitors has been developed so far, including peptides, peptide-mimetics, and small molecules, which are briefly overviewed herein.

4.1.1. Peptide and peptide-mimetic inhibitors of 14-3-3 PPIs

The first inhibitor of 14-3-3 PPI has been R18, an unphosphorylated peptide (sequence PHCVPRDLSWLDLEANMCLP) harboring the central WLDLE binding motif that, as evinced by the crystallographic structure of 14-3-3/R18 complex (PDB ID: 1A38) [109], binds to the highly conserved amphipathic groove of 14-3-3s and competes with the binding of physiological partner proteins [110]. Afterward, to enhance R18 activity, its dimeric sequence called difopein (DImeric FOurteen-three-three PEptide INhibitor) was expressed and used to investigate the physiological effects of 14-3-3/partner protein binding in cells [111, 112]. These studies demonstrate for the first time that the physiological functions of 14-3-3 could be modulated by means of specific PPIs inhibitors. Even though the development of R18 or difopein as therapeutic agents may be hampered by their chemically labile nature and poor pharmacokinetics, these peptides set the proof-of-concept for the development of therapeutic agents targeting 14-3-3 PPIs. Nowadays, R18 and difopein are still used as reference tool inhibitors of 14-3-3 PPI in many biological or chemical biology studies aimed at clarifying the role of 14-3-3 in different cells or tissues [113, 114], or as tools to prove the efficacy of new screening methods for 14-3-3 PPIs inhibitors [115, 116].

In 2010, the group of Yao reported the discovery of some peptide-mimetic 14-3-3 PPIs inhibitors by using the small molecule microarray coupled with fragment-based synthesis method [97]. Particularly, based on the optimal 14-3-3 inhibitor sequence RFRpSYPP, a hybrid library of peptide-mimetics was generated and screened for 14-3-3 binding. A good affinity towards 14-3-3σ was observed for five compounds, which were subsequently used as starting fragments to generate six recombinant peptide-mimetics. Among them, the molecule named **2-5** (Figure 5) showed the highest affinity toward 14-3-3σ, with an IC_{50} of 2.6 μM [97].

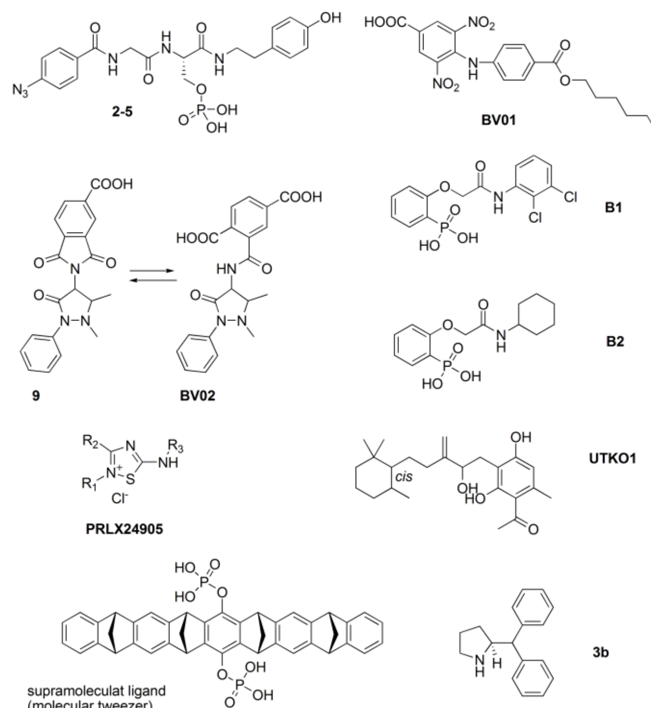

Figure 4. Chemical structure of peptide-mimetic and small molecule non-covalent inhibitors of 14-3-3 PPIs.

4.1.2 Non-covalent small molecule inhibitors of 14-3-3 PPIs

In 2010, our research group identified the first non-peptidic or peptide-mimetic inhibitors of 14-3-3 PPIs by using molecular modeling tools. Particularly, a simplified pharmacophoric model was built based on key structural features highlighted in a crystallographic complex of 14-3-3σ (PDB ID: 1YWT) [24]. Such pharmacophore model was subsequently used as query to filter a library of commercially available small molecules (the Asinex screening library). Putative hits selection was refined by applying a molecular docking protocol, and 14 small molecules were selected and submitted to biological tests. A proliferation assay on Ba/F3 cells identified **BV02** (Figure 5) as the most promising 14-3-3 PPIs inhibitor among investigated molecules, while further experiments highlighted that **BV02** inhibited 14-3-3/c-Abl interaction at low micromolar concentration [45, 107]. Interestingly, **BV02** has provided comparable antiproliferative effects also in cells expressing the Imatinib-resistant T315I Bcr-Abl construct, while promoting c-Abl nuclear relocation *in vitro*. These results clearly indicate that **BV02** could represent a valid option for the treatment of CML, particularly of Imatinib-resistant forms [107]. Since then, **BV02** has been used as reference inhibitor of 14-3-3 PPIs in many researches [117-120]. However, a hydration/dehydration pathway that generates the corresponding phthalimide derivative **9** and *vice versa* (Figure 5) was recently identified as the possible mechanism of **BV02** chemical instability. To prove this hypothesis, and to understand what is the bioactive form of **BV02** that interacts with 14-3-3 in cells,

we first performed some molecular modeling studies showing that both **BV02** and **9** are able to bind the 14-3-3σ amphipathic groove in a comparable manner and to bind the same pattern of 14-3-3 residues [47]. Afterwards, we tested the newly designed and synthesized **9** in the context of a cellular screening, in a c-Abl relocation assay, as well as in cancer and multi-drug resistant cancer cells, providing results that are highly comparable to those obtained for **BV02** [47]. Taken together, these evidences suggest that **9** is the bioactive form of **BV02** *in vitro*. To further corroborate these findings, recently we took advantage of nuclear magnetic resonance (NMR) to monitor the conversion of **9** to **BV02** in solution, and in the presence of recombinant 14-3-3σ [96]. Results of this study clearly indicate that, contrarily to **BV02**, its phthalimide derivative **9** binds to the 14-3-3σ protein, thus becoming the bioactive form. The interaction with the macromolecular target prevents the degradation of **9** to **BV02**, which is instead promoted by prolonged time of incubation and high pH values [96].

Overall, in these works computational modeling emerged as a versatile and valuable tool to design and screen 14-3-3 PPIs inhibitors [46]. Accordingly, high throughput molecular docking was used in combination with pharmacophore screening and conventional molecular docking simulations in the attempt to enhance chemical diversity of previously selected hits, and to improve their inhibitory efficacy. Indeed, only compounds having a theoretical affinity to 14-3-3σ higher than **BV02** were selected for i*n vitro* tests, which led to the discovery of the small molecule **BV01** (Figure 5) as a new micromolar inhibitor of 14-3-3σ PPIs. Notably, the direct interaction of **BV01** to recombinant 14-3-3σ was confirmed by tr-NOEs NMR experiments [46].

The group of Ottmann, which largely contributed to the state of the art of structural analysis of 14-3-3, identified in 2013 some new small-molecule inhibitors of 14-3-3 PPIs by applying a two-round virtual screening protocol [95]. From the first screening, two molecules were identified, possessing a common scaffold based on the phosphonate group. Subsequently, the exploration of this validated scaffold allowed to define structure-activity relationships and led to the identification of **B1** and **B2** (Figure 5) as the most potent 14-3-3 PPIs inhibitors of this series, which showed IC_{50} values of 5 μM and 15 μM, respectively. Notably, structural details of the interaction between these molecules and 14-3-3σ have been elucidated for the first time by X-ray crystallography (Figure 6), thus providing hints for further structure-guided optimization, as well as corroborating and validating previous computational studies. **B1** and **B2** have been found active in a cell-based assay and, due to their low membrane permeability, were classified as the first specific non-covalent inhibitors of extracellular 14-3-3 PPIs.

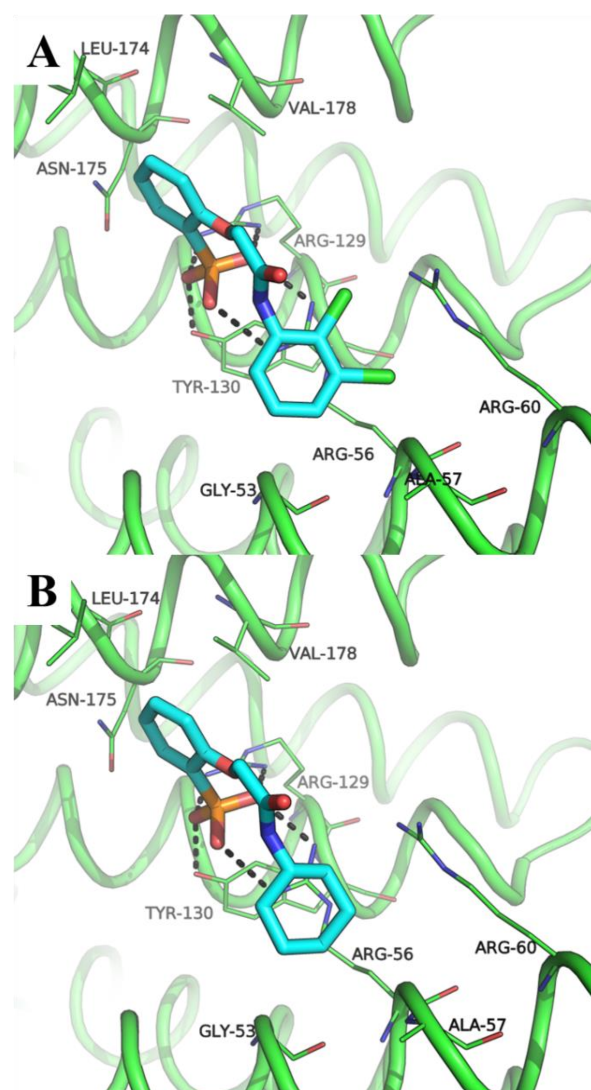

Figure 6. Crystallographic binding mode of B1 (A) and B2 (B) towards 14-3-3σ (PDB ID: 4DHU and 4DHT, respectively). 14-3-3 residues (green lines and cartoon) within 4 Å from each crystallographic ligand (cyan sticks) are showed and labeled. Polar contacts are highlighted as black dashed lines [95].

The interaction between 14-3-3 and the heat-shock protein 20 (HSP20) was identified as the possible molecular target for the discovery of small molecule against airflow obstruction in asthma [121]. In this respect, a high-throughput fluorescence polarization assay was developed and used to screen a drug-like chemical collection. Although chemical structures were not fully disclosed by the authors, compounds bearing the 1,2,4-thiadiazole substructure (**PRLX24905**, Figure 5) have been among the most promising molecules *in vitro* and *ex vivo*, thus encouraging the use of this chemically accessible scaffold to develop anti-asthma therapeutics targeting 14-3-3 [121]. In an effort to optimize the natural product moverastin-A [122], which acts as weak farnesyltransferase inhibitor in EC17 cells, and to further characterize it mechanism of action, the chemical

derivative **UTKO1** was designed and synthesized (Figure 5) [123]. Unexpectedly, **UTKO1** was unable to inhibit farnesyltransferase, despite its noticeably higher potency than moverastin-A in inhibiting the migration of cancer cells. *In vitro* experiments have then been conducted to identify the biomolecular target of **UTKO1**, clearly showing that the molecule binds to 14-3-3ζ (even if authors did not exclude the interaction with other 14-3-3 isoforms). Particularly, **UTKO1** proved to be an inhibitor of the PPIs between 14-3-3 and Tiam1, which is crucial for the second wave of Rac1 activation, thus resulting in the inhibition of cell migration [123].

An interesting example of unconventional 14-3-3 PPIs inhibitors was reported by Ottmann and co-workers in 2013 [124], who highlighted the capability of a supramolecular ligand (molecular tweezers, Figure 5) to bind specifically a lysine residue of 14-3-3 with a K_d of 20 μM. Due to the biological relevance of 14-3-3 proteins in the cellular environment and due to the fact that 14-3-3σ contains 17 lysine residues, the direct binding of tweezers to 14-3-3σ was measured by using isothermal titration calorimetry. Further, fluorescein-labelled synthetic peptides were used to investigate the influence of tweezers on 14-3-3/partner protein binding (in a phosphorylate and unphosphorylated manner). The supramolecular ligand inhibits the interaction between14-3-3σ and partner protein in a concentration dependent manner. To obtain the structural detail of interaction, the crystallographic structure of 14-3-3σ in complex with molecular tweezers was solved, showing the tweezers molecule bound to a surface-exposed lysine (Lys214) at the beginning of the last C-terminal helix of 14-3-3σ (Figure 7). The binding mode of tweezers was also corroborated by molecular dynamics studies.

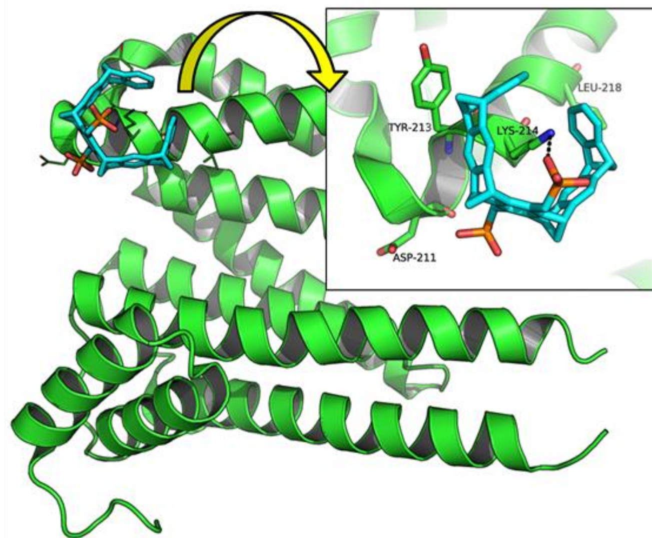

Figure 7. Crystallographic binding mode of the supramolecular ligand (molecular tweezers) towards 14-3-3σ (PDB ID: 4HQW). 14-3-3 residues within 4 Å from the crystallographic ligand are showed as lines and are labeled [124].

Recently, a rational design of 14-3-3 PPIs inhibitors has been conducted by the group of Ottmann, guided by the structure-based analysis of 14-3-3 PPIs stabilizers (see below). In searching for candidate therapeutic against Alzheimer disease, authors have designed and optimized a series of low-molecular weight inhibitors of 14-3-3 PPIs using the Tau epitope[125], as non-covalent tether to occupy simultaneously the amphipathic groove and the stabilizers binding site of 14-3-3. Indeed, this strategy has originated by the observation of X-ray crystallography structures, showing a clear overlap between the binding of Tau peptide and Fusicoccin A to 14-3-3 [126]. By combining organic synthesis with X-ray crystallography and biochemical evaluation, authors disclosed 3b (Figure 5) as the most potent 14-3-3 PPIs inhibitor of the series, capable of affecting the binding of full-length Tau to 14-3-3 (IC50 = 5.9 μM).

4.1.3 Covalent small molecule inhibitors of 14-3-3 PPIs

In 2011, the group of Fu identified **FOBISIN101** (FOurteen-three-three BInding Small molecule Inhibitor) as the first covalent inhibitor of 14-3-3 [98]. Particularly, they used a fluorescence polarization-based binding assay to screen the LOPAC library in search of compounds able to disrupt the 14-3-3/phosphopeptide interaction. Among tested molecules, **FOBISIN101** (Figure 8) emerged as a pan-inhibitor of all 14-3-3 isoforms, and it was able to impair the binding of both phosphorylated and unphosphorylated 14-3-3 partner proteins.

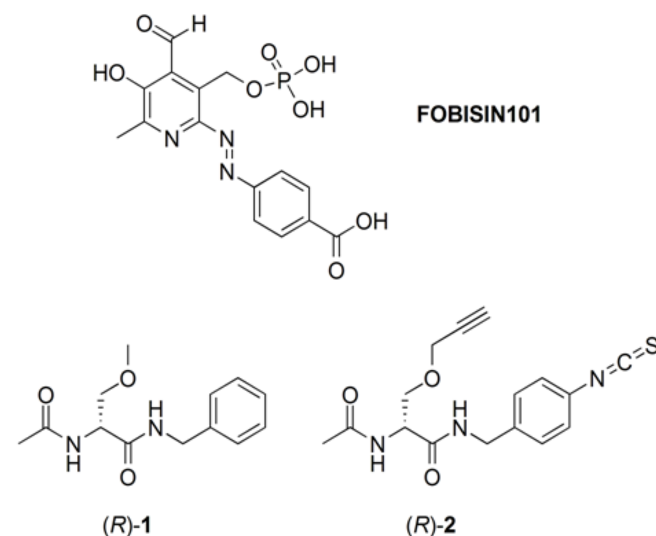

Figure 8. Chemical structure of small molecules covalent inhibitors of 14-3-3 PPIs.

The crystallographic structure of **FOBISIN101** in complex with 14-3-3 protein (PDB ID: 3RDH, Figure 9) revealed that **FOBISIN101** binds in a covalent manner to Lys120 in the 14-3-3 amphipathic binding groove. Particularly, the N=N double bond that links the pyridoxal-

phosphate to the benzoate moiety in **FOBISIN101** was reduced by X-ray radiation, and the pyridoxal-phosphate moiety was covalently bound to the side chain of Lys120 (Figure 9). Accordingly, **FOBISIN101** and its derivatives have been proposed as radiation-triggered inhibitors of 14-3-3 PPIs, even if, to date, further developments of this class of potential therapeutics have not been reported.

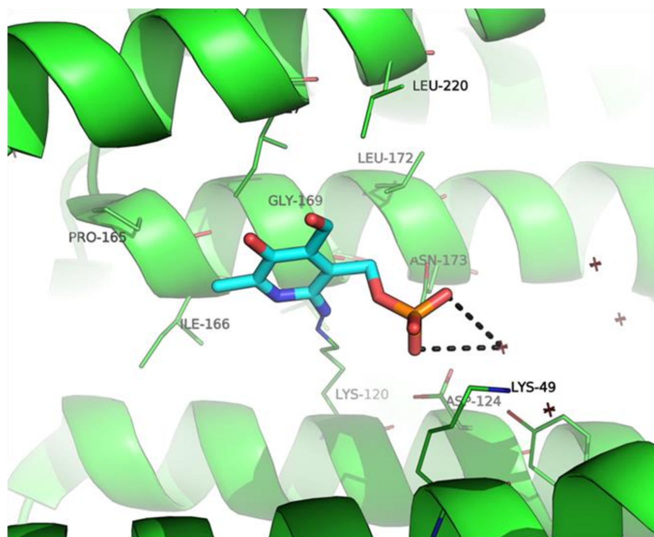

Figure 9. Crystallographic binding mode of FOBISIN101 (cyan sticks) towards 14-3-3ζ (green lines and cartoon; PDB ID: 3RDH). 14-3-3 residues within 4 Å from the crystallographic ligand are showed as lines [98]. Polar contacts are highlighted by black dashed lines.

The anti-epileptic drug lacosamide (Figure 8) was used as molecular probe in an assay based on affinity bait (AB) and chemical reporter (CR) group (AB&CR assay), aimed at identifying its possible macromolecular target. In this work from Park et al. [116], lacosamide (*R*)-**1** was chemically modified by the addition of an isothiocyanate AB and a propargyl CR group to originate (*R*)-**2** (Figure 8). By fishing in a soluble lysate of rodent brain, authors have found that (*R*)-**2** binds preferentially to 14-3-3ζ in a covalent manner, through anchoring the Lysine 120 which is located near the amphipathic groove. Surprisingly, this interaction was dependent from the presence of xanthine, a purine base relatively abundant in human tissues and fluids, which proved to bind the recombinant 14-3-3ζ with noticeable affinity by ITC measurements. Although no direct evidences on the inhibition of 14-3-3 PPIs by these agents have been provided, (*R*)-**1** and xanthine proved valuable starting points for further investigations [116].

4.2 Stabilizers of 14-3-3 PPIs

14-3-3 have been reported to increase the stability of some physiologically-relevant proteins that are deregulated in a pathological context. In that cases, the use of molecules that are able to further stabilize the 14-3-3/partner protein interactions is thought to increase partners' functional expression [106]. Compared to PPIs inhibitors, the design of stabilizers is effectively more complex since these molecules are expected to bind simultaneously to 14-3-3 and its partner protein. Nevertheless, some examples of natural products stabilizers of PPIs are known and could help the research in this direction [106]. Moreover, based on the higher molecular complexity of the system targeted by PPIs stabilizers than single 14-3-3, this class of 14-3-3 modulators is thought to be selective for specific 14-3-3 isoforms.

The two main classes of 14-3-3 PPIs stabilizers developed so far, namely natural and non-natural compounds, are briefly reviewed here below.

4.2.1 Natural products as stabilizers of 14-3-3 PPIs

Fusicoccin (**FC**, Figure 10) is a toxin produced by the fungus *Phomopsis amygdali* that activates the plant plasma membrane H^+-ATPase (bearing a mode-3 14-3-3-binding phosphopeptide) thus affecting numerous physiological and biochemical processes of higher plants [127, 128]. **FC** binds the already formed 14-3-3/H^+-ATPase complex, and stabilizes this interaction by two orders of magnitude thus maintaining the enzyme in its activated form [127]. A number of crystallographic structures of the ternary complex **FC**/14-3-3/H^+-ATPase have been solved to date [127, 129], allowing the clarification of molecular basis of this interaction and also providing structural explanation to the **FC** inability to bind 14-3-3 in complex with mode-1 or mode-2 phosphopeptides (Figure 11A) [128].

Indeed, they occupy partially the **FC** binding pocket in the 14-3-3 amphipathic binding groove, thus competing with **FC** itself. The recent discovery that **FC** could stabilize 14-3-3 PPIs also in complexes with other mode-3 phosphopeptides like the Glycoprotein Ibα [130], opens a new scenario in **FC** research and its potential role in the treatment of human diseases. Furthermore, **FC** has been successfully used to stabilize additional 14-3-3/protein complexes, and several structural details on these interactions have been provided [105, 106, 131].

Cotylenin A (**CN-A**, Figure 10) is a metabolite produced by the fungus *Cladosporium* and is chemically related to **FC**. **CN-A** is able to bind the plant plasma membrane H+-ATPase protein, and to induce differentiation in human acute myeloid leukemia in both cell cultures and mouse models [133], but these effects are not observed in **FC**. The crystallographic structure of the ternary complex **CN-A**/14-3-3/H^+-ATPase (Figure 11B, PDB ID: 3E6Y) [132], provides a possible explanation for the functional differences observed between **FC** and **CN-A**. Indeed, the steric conflict between **FC** and mode-1 and -2 phosphopeptides seems not to exist in the case of **CN-A**, due to the absence of the C12 hydroxyl group. Notably, this side of the stabilizers that points towards the amphipathic groove has been recently exploited in the rational design of 14-3-3 PPIs (see above) [39].

Mizoribine (**MIZ**, Figure 10) is an imidazole nucleoside first isolated from the culture media of *Eupenicillum brefaldianum* in Japan [134], where it is used for

immunosuppressive therapy after renal transplantation, for lupus nephritis and for rheumatoid arthritis. It has been demonstrated that **MIZ** can affect 14-3-3 stability by enhancing its interaction with glucocorticoid receptor *in vitro* in a dose-dependent manner. This mechanism of action could explain, at least partially, the pharmacological effects of **MIZ** [134], even if structural details or deeper studies have not been performed on this molecule.

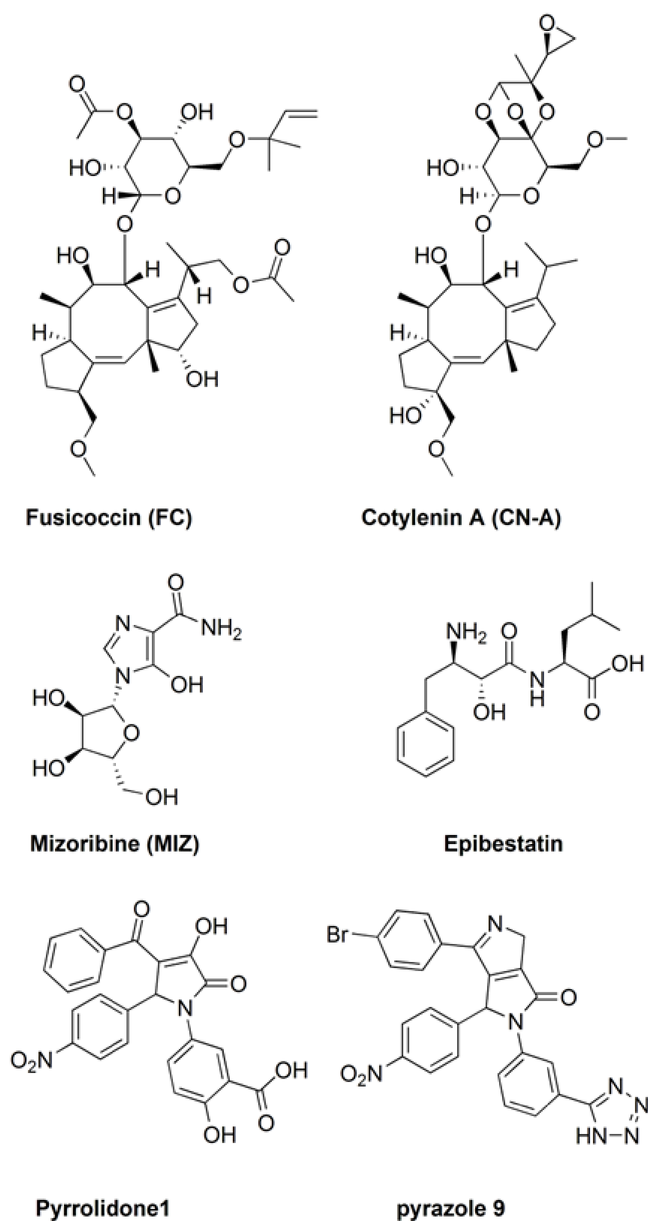

Figure 10. Chemical structure of small molecule stabilizers of 14-3-3 PPIs.

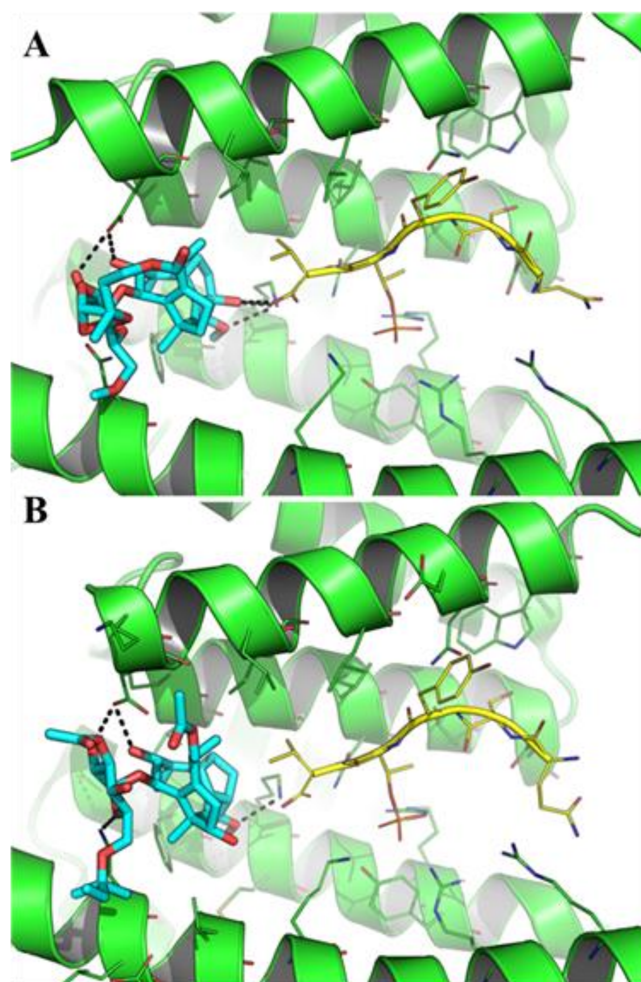

Figure 11 Crystallographic binding mode of Fusicoccin (A) and Cotylenin A (B) towards the 14-3-3/H+-ATPase complex (PDB IDs: 1O9F and 3E6Y, respectively)[127, 132]. 14-3-3 residues (green lines and cartoon) within 4 Å from the crystallographic ligand (cyan sticks) and the phosphorylated peptide (yellow lines and cartoon) are showed as lines. Polar interactions are highlighted by black dashed lines.

4.2.1 Non-natural products as stabilizers of 14-3-3 PPIs

Epibestatin and **Pyrrolidone1** (Figure 10) have been the first non-natural stabilizers of 14-3-3/plasma membrane H+-ATPase complex ever discovered by means of high throughput screening of a compounds library[48]. Their binding mode has been elucidated by X-ray crystallography (Figure 12) showing that the molecules bind and stabilize the 14-3-3/plasma membrane H+-ATPase complex in a different manner. Even though they are weaker stabilizers than the naturally occurring **FC** or **CN-A**, these compounds proved to be rather specific in stabilizing a selection of 14-3-3 PPIs, thus becoming interesting starting point for the development of therapeutic agents [105]. In 2012, the medicinal chemistry optimization of the **Pyrrolidone1** scaffold has led to the identification of the more rigid **pyrazole 9** (Figure 10) that

can deeply bind to 14-3-3/H+-ATPase complex interface with an improved stabilizing efficiency [106, 135].

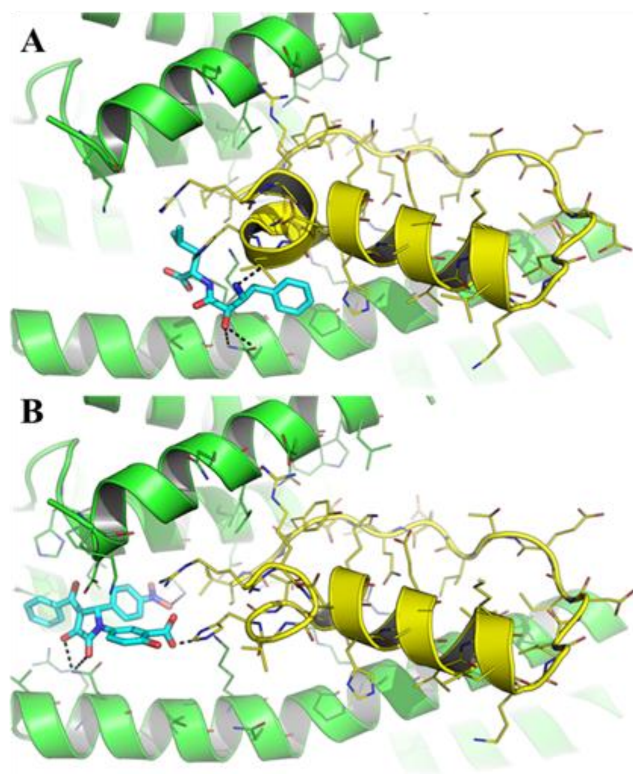

Figure 12. Crystallographic binding mode of Epibestatin (A) and Pyrrolidone1 (B) towards the 14-3-3/H+-ATPase complex (PDB IDs: 3M50 and 3M51, respectively). 14-3-3 residues within 4 Å from the crystallographic ligand and the phosphorylated peptide are showed as lines [48, 132].

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CONCLUSION

14-3-3 proteins are a family of regulatory proteins found in all eukaryotes that express different isoforms having different roles and functions. 14-3-3 activity is dependent on the interaction with a huge number of protein partners, and they control both healthy and pathological processes. Among them, the role played by 14-3-3 PPIs in cancer and neurodegenerative diseases, such as AD and PD, is well recognized. On this regards, the need of successful treatments for cancer and neurodegeneration has inspired many scientists worldwide to investigate 14-3-3 PPIs either cancer or neurodegeneration, and to discover small molecules able to modulate any aberrant associations leading to unhealthy condition.

According to the role played by 14-3-3 proteins in both diseases, potential effective drugs may inhibit or stabilize 14-3-3 PPIs. Inhibition occurs when small molecules bind to the amphipathic groove of 14-3-3 proteins and prevent their interaction with specific partner proteins, while stabilization is observed when small molecules are able to bind and stabilize the complex between 14-3-3 and the partner.

Many evidences suggest that targeting 14-3-3 PPIs by small molecules represents a promising strategy for the treatment of several pathologies including cancer and neurological disorders. Despites efforts reviewed herein, potent and specific 14-3-3 PPIs inhibitors or stabilizers are still urgently needed not only as preclinical candidates for pharmaceutical development, but also as chemical biology tools for further understanding the role of this protein family in cell processes and human diseases. Nevertheless, small molecule modulators of 14-3-3 PPIs available to date represent a valuable starting point for medicinal chemistry-oriented optimization, which should aim at improving potency and drug-likeness of these lead structures.

LIST OF ABBREVIATIONS

PPIs Protein-Protein Interactions

CML Chronic Myeloid Leukemia

AD Alzheimer’s disease

PD Parkinson disease

TSEs Transmissible Spongiform Encephalopathies (TSEs)

CJD Creutzfeldt-Jakob disease

LBs Lewy Bodies

NFTs neurofibrillary tangles

CSF cerebrospinal fluid

WHO World Health Organization

TH tyrosine hydroxylase

FOXO3a forkhead box transcription factor, class O

LRRK2 leucine-rich repeat kinase 2

NMR Nuclear Magnetic Resonance

FOBISIN101 FOurteen-three-three BInding Small molecule Inhibitor

AB Affinity Bat

CR Chemical Reporter

FC Fusicoccin

CN-A Cotylenin A

MIZ Mizoribine

difopein DImeric FOurteen-three-three PEptide Inhibitor

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