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14-3-3 proteins as potential therapeutic targets

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Abstract

The 14-3-3 family of phosphoserine/phosphothreonine-binding proteins dynamically regulates the activity of client proteins in various signaling pathways that control diverse physiological and pathological processes. In response to environmental cues, 14-3-3 proteins orchestrate the highly regulated flow of signals through complex networks of molecular interactions to achieve well-controlled physiological outputs, such as cell proliferation or differentiation. Accumulating evidence now supports the concept that either an abnormal state of 14-3-3 protein expression, or dysregulation of 14-3-3/client protein interactions, contributes to the development of a large number of human diseases. In particular, clinical investigations in the field of oncology have demonstrated a correlation between upregulated 14-3-3 levels and poor survival of cancer patients. These studies highlight the rapid emergence of 14-3-3 proteins as a novel class of molecular target for potential therapeutic intervention. The current status of 14-3-3 modulator discovery is discussed.

Keywords

14-3-3 inhibitor; stabilizer

The 14-3-3 protein family represents the prototype of a class of phosphoserine/ phosphothreonine (pSer/Thr)-recognition proteins [1–5]. Analogous to Src-homology 2 domains that bind to phosphotyrosine in target proteins [6], 14-3-3 proteins can directly bind pSer or pThr residues in specific sequence contexts [7–8]. This feature allows 14-3-3 proteins to bind a multitude of functionally diverse molecules, including kinases, phosphatases, transmembrane receptors and transcription factors [1–4]. 14-3-3 proteins form homo- or heterodimers, which may impact specific client protein recognition [9]. Through these interactions, 14-3-3 proteins play important roles in a wide range of vital physiological

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and pathological processes. As such, 14-3-3 proteins are tightly controlled in normal cells and often dysregulated in a broad spectrum of human diseases [1]. Here, we briefly review the connection between 14-3-3 and various human diseases, mechanisms that regulate 14-3-3 expression and activity, and the current status of 14-3-3 modulator development. Readers are referred to a number of excellent earlier reviews for further information [4, 10–11].

1. 14-3-3 in human diseases

1.1 Cancer

Accumulating evidence from cell and animal models, as well as patient samples, has established a strong link between 14-3-3 and many types of cancer [4, 12–13]. A critical role of 14-3-3 proteins in cancer has been extensively studied particularly in breast, lung and head and neck cancers.

14-3-3 proteins support the action of oncogenes and inhibit tumor suppressors in breast cancer cells through the elaborate control of 14-3-3 protein expression patterns and regulation of the interaction of 14-3-3 with client proteins. 14-3-3 σ , a suggested tumor suppressor, is silenced in cancer patient specimens due to frequent gene methylation [14–15]. Thus, the methylation state of 14-3-3 σ can be used as a diagnostic marker. One mechanism that may explain the potential tumor suppressor activity of 14-3-3 σ involves its interaction with a key survival kinase, Akt [16]. In stark contrast to the tumor suppressor functions of 14-3-3 σ , the ζ , β and τ isoforms of 14-3-3 have been reported to be oncogenic. These isoforms were found to be overexpressed in cancer cells, as well as patient samples [17–20]. In support of a pro-oncogenic role of 14-3-3, high expression of 14-3-3 ζ is associated with poor prognosis of breast cancer patients [17]. Mechanistically, 14-3-3 ζ binds to the p85 regulatory subunit of PI3 kinase, leading to Akt activation and enhanced cancer cell survival [21], in addition to inactivating tumor suppressors like p53 and p21 [22–23]. Interestingly, 14-3-3 ζ also cooperates with ErbB2 to promote the progression of ductal carcinoma *in situ* to invasive breast cancer by inducing epithelial-mesenchymal transition and with LAPTM4B to promote chemo-resistance [24–25]. Conversely, downregulation of oncogenic 14-3-3 isoforms attenuates characteristics of tumor progression, including metastasis, chemoresistance and recurrence. Thus, therapeutically inhibiting 14-3-3 ζ , β and τ would be expected to benefit cancer patients.

Several isoforms of 14-3-3 have been linked to lung cancer development with strongest evidence supporting the involvement of 14-3-3 ζ . 14-3-3 ζ has been suggested to be a potential prognostic marker and a therapeutic target [26–28]. It is overexpressed in tumors of patients with non-small cell lung cancers (NSCLC). Knockdown of this isoform dramatically impaired anchorage-independent growth of lung cancer cells and sensitized these cells to cisplatin. Although a clear connection between 14-3-3 ζ and lung cancer has been established, the role of other 14-3-3 isoforms is complicated. While loss of function of 14-3-3 σ was correlated with metastasis, methylation of 14-3-3 σ in NSCLC patients with platinum-based chemotherapy was correlated with better survival, suggesting a possible oncogenic role for 14-3-3 σ [29–32]. Other isoforms including β , γ and τ were observed to be upregulated in cancer specimens [31, 33–34]. Therefore, further studies are required to understand the mechanisms by which each isoform of 14-3-3 contributes to tumorigenesis, so that a beneficial targeting strategy can be designed.

The correlation of 14-3-3 with head and neck cancers mainly comes from studies involving 14-3-3 ζ and σ . 14-3-3 ζ overexpression, as a result of gene amplification, was found in 30–40% of examined head and neck squamous carcinoma cases [35]. Patients with overexpression of both 14-3-3 ζ and σ exhibited a significantly decreased median disease-

free survival [36–37]. Overexpression of these two 14-3-3 isoforms was found to be a strong predictor of poor prognosis in head and neck cancers. Decreasing the expression of 14-3-3 ζ resulted in induction of apoptosis and increased sensitivity to chemotherapeutics [38–39], supporting 14-3-3 as a potential target in head and neck cancer.

Taken together, these studies indicate that members of the 14-3-3 family, especially 14-3-3 ζ , play a pro-oncogenic role in multiple tumor types. Overexpression of 14-3-3 proteins is generally associated with poor survival of cancer patients and may serve as an excellent prognostic factor.

1.2 Neurological diseases

A number of neurological diseases are associated with dysregulated 14-3-3 function. Early studies demonstrated that 14-3-3 isoforms were present in the cerebrospinal fluid of sporadic Creutzfeldt-Jakob disease [40–41] and prion disease patients [42–44]. However, the precise role of 14-3-3 in the progression of these disorders remains to be determined.

Recent research has suggested a connection between altered 14-3-3/ligand interaction and neural diseases, revealing a pathological role of 14-3-3. For example, Leucine-Rich Repeat Kinase 2 (LRRK2) protein is mutated in Parkinson's disease (PD) patients. These LRRK2 mutations result in dephosphorylation of Ser935 and 14-3-3 disassociation [45–48]. In several cellular and animal PD models, multiple isoforms of 14-3-3 have impaired binding to α -synuclein (α -syn), leading to α -syn toxicity in dopaminergic neurons [49]. An important neuroprotective role of 14-3-3 ζ was also reported, which relied on its binding to and activation of tyrosine hydroxylase. These studies link reduced 14-3-3 ζ level to PD pathology [50]. Given the accumulating data that support the involvement of 14-3-3 in PD, enhancement of 14-3-3 expression or ligand binding may offer an avenue to protect neurons from injury.

In contrast to the decreased 14-3-3/client protein interaction that is observed in PD, differential 14-3-3/ligand interactions were observed during the onset of seizures [51]. 14-3-3 ζ binding to PKC δ was enhanced due to phosphorylation of 14-3-3 ζ at Ser58, which was accompanied by the release of pro-apoptotic client proteins to induce neuronal cell death. Thus, strategies for targeting the 14-3-3/PKC complex may have therapeutic significance for neuroprotection against injury after a seizure.

A role for 14-3-3 in Alzheimer's disease (AD) has also been suggested. 14-3-3 proteins have been found in neurofibrillary tangles, a major structure in AD patients. Within the neurofibrillary tangles, increased binding of 14-3-3 to Tau, a microtubule-associated protein, has been observed. This interaction is mediated by phosphorylation of Ser214 on Tau, which is significantly enhanced in the brains of AD patients. Therefore, upregulated 14-3-3/Tau interaction may contribute to AD pathology [52]. Further experimental validation will be required to confirm 14-3-3 as a therapeutic target for AD.

1.3 Other diseases

14-3-3 isoforms have also been implicated in the pathogenesis of a broad range of other diseases. For example, the role of 14-3-3 in diabetic cardiomyopathy has been suggested [53]. Evidence now is emerging that demonstrates the differential expression patterns of 14-3-3 in healthy controls versus patients with ailments such as joint inflammation [54], multiple sclerosis [55–56] and endometriosis [57]. Furthermore, studies have revealed abnormalities in signaling mediated by 14-3-3 in diseases such as glaucoma [58], phospholipidosis [59] and infectious diseases [60–61]. It appears that altered interactions between 14-3-3 and client proteins may be responsible for a variety of diseases. For example, in response to hypoxia, the 14-3-3/REDD1 interaction inhibited mTOR signaling in rats

patients with chronic obstructive pulmonary disease [62]. These studies suggest that members of the 14-3-3 family may serve as attractive therapeutic targets.

2. Regulation of 14-3-3

To manipulate the function of 14-3-3 proteins for therapeutic gains, it is necessary to understand how 14-3-3 proteins are controlled. A number of mechanisms regulate the expression and function of 14-3-3 proteins.

2.1 Expression

Extensive evidence indicates that 14-3-3 isoforms are altered at the genomic level, including epigenetic silencing and gene amplification. According to their distinct roles in disease contexts, 14-3-3 can be divided into loss-of-function and gain-of-function categories. The tumor suppressive role of 14-3-3 σ has been correlated with its epigenetic silencing [12]. In contrast, the other six isoforms, usually thought of as tumor-promoters, are upregulated to different degrees dependent on cancer types. One mechanism for this upregulation is due to gene amplification. For example, a gain in 14-3-3 ζ copy number has been observed in patient with breast and head and neck cancer [17, 35].

Another mechanism is through post-transcriptional modulation of the stability of 14-3-3 mRNA. Under physiological conditions, an emerging role for microRNAs in the regulation of 14-3-3 has been demonstrated. miRNAs are endogenous modulators that repress target gene expression and uses the same processing machinery as exogenous siRNAs that degrade the mRNAs of target genes. Several miRNAs have been shown to target 14-3-3 ζ in both cell and animal models. For example, MCF7 cells transfected with miR-193b, an upstream regulator of 14-3-3 ζ , showed reduced growth [63]. However, single knockdown of 14-3-3 ζ did not affect cell growth, suggesting that miR-193b-induced growth inhibition involves multiple targets. In a miR-375 transfected gastric cancer cell model, the 3'UTR of 14-3-3 ζ was targeted by miR-375 for silencing, which partially accounted for the observed tumor suppressive effect of miR-375. Interestingly, loss of miR-375 was observed in gastric carcinomas, indicating that miRNAs may serve as a new target for attenuating 14-3-3-mediated oncogenic pathways [64]. Strong data in support of the miRNA-14-3-3 regulatory axis came from experiments with miR-451 knockout mice. 14-3-3 ζ was an essential target for miR-451 to mediate erythropoiesis and to protect against erythroid oxidant stress [65–66]. With the list of 14-3-3 regulatory miRNA expanding, strategies to control 14-3-3 expression through manipulating miRNA will be expected to attract increased attention.

The third mechanism by which 14-3-3 expression is controlled is through proteasome-mediated degradation. For example, the stability of 14-3-3 σ is tightly regulated by an E3 ligase, Efp. Loss of 14-3-3 σ through degradation leads to uncontrolled proliferation of breast cancer cells and tumorigenesis [67]. This study supports a tumor suppressor function of 14-3-3 σ and provides a novel therapeutic strategy for targeting proteins that regulate the degradation of 14-3-3.

2.2 Function

14-3-3 proteins function primarily as adaptor proteins via binding to various client proteins. Therefore, mechanisms that control 14-3-3/client protein interactions will impact 14-3-3 function. There are two major avenues that regulate 14-3-3/client protein interactions: (i) modification of 14-3-3 proteins and (ii) modification of client proteins at 14-3-3 binding motifs.

2.2.1. Modification of 14-3-3 proteins—A key mechanism for post-translational regulation of 14-3-3 is through phosphorylation. Several upstream kinases are involved in phosphorylation-mediated modulation of 14-3-3. The functional impact of such kinases on the client protein binding capacity of 14-3-3 has been reviewed [68]. Evidence suggests that phosphorylation of 14-3-3 proteins may reduce their interaction with client proteins or indirectly interfere with the binding of 14-3-3 to client proteins through disruption of 14-3-3 dimers.

14-3-3 ζ and β can be phosphorylated at S184 and T223. Phosphorylation of S184, in part through JNK, has been reported to reduce the affinity of 14-3-3 to phosphorylated Bad, resulting in the release of Bad which then induces apoptosis [69]. In addition, T223 of 14-3-3 can be phosphorylated by casein kinase 1 α , which results in the disruption of the interaction between 14-3-3 and Raf [70]. The complexity of phosphorylation-mediated regulation of 14-3-3 is undoubtedly intricate and thus requires further investigation.

The interaction of 14-3-3 with client proteins depends on the dimerization status of 14-3-3 proteins. The seven 14-3-3 isoforms exist in a dynamic balance of homo- or heterodimers [71–72]. It is well established that the N-terminal fragment of 14-3-3 regulates dimerization. In particular, Ser59 of 14-3-3 ζ is functionally critical for the formation of dimers [68]. Phosphorylation of Ser59 at the dimerization interface of 14-3-3 results in the disassociation of 14-3-3 dimers and reduction of client protein binding. Therefore, modulation of Ser59 phosphorylation could serve as a mechanism to regulate 14-3-3 dimerization and thus the ability of 14-3-3 to interact with client proteins.

14-3-3 proteins can also be modified by additional mechanisms, including acetylation. This topic is extensively covered in other chapters (Aitken).

2.2.2 Modification of client proteins—A distinguishing feature of 14-3-3 proteins, as regulators of multiple cellular processes, is their ability to bind numerous functionally diverse partners. This ability is determined by the presence of a common structure in various client proteins as defined as 14-3-3 protein recognition motifs [2]. A primary mode of interaction of 14-3-3 with a client protein is dictated by phosphorylation of client proteins at defined 14-3-3 recognition motif(s). As detailed in other chapters of this volume (Obsil), three such motifs have been described, with RSxpS/TxP being the prototype motif. Thus, phosphorylation of such motifs in client proteins controls the dynamic interaction of 14-3-3 proteins with these modified partners, subjecting the 14-3-3/client protein interaction to the control of cellular kinase and phosphatase signaling networks. In this way, environmental cues or pathological perturbations, such as oncogenic activation, of signaling pathways that influence phosphorylation of client proteins are expected to impact the function of 14-3-3 proteins.

3. Development of 14-3-3 modulators for chemical biology and drug discovery

The mechanistic understanding of 14-3-3 regulation allows for a number of strategies to manipulate 14-3-3 for potential therapeutic applications. It is envisioned that approaches can be developed to control the expression and stability of 14-3-3 proteins, dimerization of 14-3-3, and 14-3-3/client protein interactions. The expression levels of 14-3-3 proteins may be perturbed through modification of the transcriptional, epigenetic machinery of 14-3-3 isoforms or through miRNA. Manipulation of the protein degradation system for 14-3-3 may impact the half-life of intracellular 14-3-3 isoforms. In addition, interference of the activity of kinases and phosphatases that are specific to a 14-3-3 phosphorylation site may be used to influence 14-3-3 dimerization and/or client protein association. Because 14-3-3 dimerization

is important for client protein binding, tools that disrupt the dimer interface may be developed to decrease 14-3-3 function. Although above approaches seem feasible, current efforts focus largely on targeting 14-3-3/client protein interactions for potential tool discovery and therapeutic development.

3.1. Methods to monitor 14-3-3/client protein interactions

In order to discover 14-3-3 protein modulators, it is essential to develop highly sensitive methods to monitor the specific interaction of a 14-3-3 protein with client proteins. There are a number of powerful protein-protein interaction technologies that have been implemented for the study of 14-3-3 proteins, such as virtual screening, chemical microarray and isothermal titration calorimetry [73–75]. Here, we briefly review techniques that are particularly suited for high-throughput screening (HTS). An assay suitable for HTS requires target specificity, robust readout, day-to-day and plate-to-plate reproducibility, technical simplicity, suitability for automation and low cost. Several HTS assays have been developed for monitoring the interaction of 14-3-3 with its client proteins, including fluorescence polarization (FP) [76–77], AlphaScreen [78], time-resolved fluorescence energy transfer (TR-FRET) [79] and label-free biosensor assays [80].

3.1.1. Fluorescence polarization assay—FP is a highly sensitive method for the study of molecular interactions in solution [81]. This method can be used to measure the interaction of two molecules if one of the molecules is relatively small and fluorescent. When fluorescent small molecules (such as tagged peptides) are bound by large molecules (such as a protein), the movement of the complex becomes slower. Thus, the binding of a fluorescently labeled peptide to a protein can be monitored by the change in polarization. FP-based assays are widely used for HTS due to their simplicity and sensitivity, which was applied to the 14-3-3/client interaction. It was designed and optimized for monitoring the association of 14-3-3 with a rhodamine-labeled Raf-1 phosphopeptide [76]. This solution-based assay demonstrated a robust performance with a signal-to-noise ratio > 10 and a Z' factor > 0.7 . An alternative assay with a carboxyfluorescein-labeled 14-3-3 binding peptide was developed for the same purpose [77]. Again, this assay exhibited robust performance, suitable for HTS.

3.1.2. AlphaScreen assay—AlphaScreen (Amplified Luminescent Proximity Homogenous Assay) technology provides a proximity-dependent homogenous assay format for studying molecular interactions [82–83]. This assay system contains two beads, a donor bead and an acceptor bead. Donor beads contain the photosensitizer phthalocyanine, which, upon illumination at 680 nm, converts ambient oxygen to an excited form of oxygen, singlet oxygen. If an acceptor bead is within the energy diffusion range (< 200 nm) in solution, energy is transferred from the singlet oxygen to thioxene derivatives within the acceptor bead, resulting in light production (520–620 nm). AlphaScreen technology offers a bead-based, non-radioactive homogenous assay that can be adapted to small volumes in a microplate format. Using both phosphorylated and nonphosphorylated client proteins of 14-3-3, we have designed and optimized an AlphaScreen-based 14-3-3 assay for HTS [78]. By utilizing the interaction of 14-3-3 with a phosphorylated Raf-1 peptide and a nonphosphorylated R18 peptide as model systems, we have established a homogenous "add-and-measure" HTS assay. Both assays achieved robust performance with S/B ratios above 7 and Z' factors above 0.7. Application of known antagonistic peptides further validated the assay for screening of chemical compound libraries to identify small molecules that can modulate 14-3-3 protein-protein interactions.

3.1.3. Fluorescence resonance energy transfer (FRET) assay—FRET is a non-radioactive, photophysical effect in which energy that is absorbed by a donor fluorophore is

transferred to an acceptor fluorescent molecule. It requires paired fluorophores with overlapping donor emission and acceptor excitation spectra. By coupling the donor and acceptor fluorophores to two interacting proteins, the fluorophores may be brought into close proximity, generating a FRET signal. The use of a donor fluorophore with a long half-life, such as europium, allows a time-delayed (typically 50–150 μ s) measurement of FRET signal; thus, this method is known as time-resolved FRET (TR-FRET). TR-FRET is especially suitable for compound screening as it minimizes readout interference from short-lived background fluorescence, such as media or small molecule autofluorescence.

To screen for small molecules that specifically inhibit the interaction between 14-3-3 ζ and Bad, we have developed and validated a TR-FRET HTS assay (PubChem Assay ID 781) [79]. This homogenous assay has been miniaturized and optimized for ultra-HTS (uHTS) in a 1536-well format. In this assay, hexahistidine (6xHis)-tagged 14-3-3 ζ protein is indirectly labeled with a europium chelate using a europium-conjugated anti-6xHis antibody, while a phospho-peptide derived from Bad is directly labeled with Dy647. Interaction of 14-3-3 ζ protein and phospho-Bad peptide brings the two fluorophores into proximity, leading to an energy transfer from europium to Dy647 and generation of a FRET signal. This assay is robust with a consistent Z' factor of 0.6–0.9, allowing it to be used for screening of 14-3-3 small molecule inhibitors.

3.1.4. Label-free optical biosensor assay—Label-free detection technologies have long been recognized for their ability to probe biomolecular interactions without the need for labeling target molecules [84–85]. Among a variety of label-free technologies, such as surface plasmon resonance, the use of optical biosensors in a plate format for monitoring biomolecular interaction is gaining increased attention [86]. One such a label-free optical biosensor technology is Corning's Epic system, which is based on the principle of resonant waveguide grating (RWG) [85–87]. Sensors on the microplate are coated with a surface chemistry layer that enables covalent attachment of biomolecules. Binding of partners to the immobilized target induces a shift in the wavelength of light that is reflected from the sensor. The magnitude of this wavelength shift is proportional to the amount of analyte that binds to the immobilized target.

Using the Epic System, a label-free assay for phospho-specific interactions of 14-3-3 has been developed in a 384-well HTS format [80]. A NH₂-SWpTY peptide, which interacts with 14-3-3, was covalently immobilized on the surface of a sensor plate. Binding of 14-3-3 protein to immobilized NH₂-SWpTY peptides leads to the generation of RWG signal over the reference. The specificity of the assay has been validated using competition experiments with a non-phosphorylated SWTY peptide and a binding mutant of 14-3-3 protein. This label-free optical biosensor assay is robust with Z' above 0.5 and can be applied for the screening of modulators of 14-3-3 protein–protein interactions.

Together, these tested assays provide practical tools for quantifying the impact of modulators on 14-3-3/client protein interactions. Effective application of these assays is expected to accelerate 14-3-3 modulator discovery.

3.2. 14-3-3/client protein interaction modulators

3.2.1. Peptide inhibitors of 14-3-3—To identify 14-3-3 modulators, phase display technology was employed to discover peptides that can bind to 14-3-3 proteins [88]. Several peptides were obtained, among which R18 has been the most studied and demonstrates a potent effect in disrupting 14-3-3/client protein interactions (Fig. 1). Structural analysis revealed that the R18 sequence mimics the phosphorylated 14-3-3-recognition peptide for docking in the amphipathic groove of 14-3-3, the site for the binding of natural partners [89–90]. Thus, R18 competes with natural client proteins for binding to the same site on 14-3-3,

explaining its potent 14-3-3 inhibitory effect. To further enhance R18 activity in cells, a dimeric R18 sequence, termed difopein (Fig. 1), was expressed and used to probe the function of 14-3-3/client protein interactions in cell physiology [91]. Such studies have led to the clear demonstration of a crucial role for 14-3-3 proteins in supporting cell survival as expression of R18, or difopein, induces apoptotic cell death.

The well characterized R18 peptide and difopein have been extensively utilized as powerful tools for dissecting the role of 14-3-3 in a variety of physiological and pathological processes [10, 92–94]. Applications of R18 and difopein range from cell-free biochemical systems, cell lines, to animal models, for 14-3-3 functional studies and potential therapeutic interventions. For example, R18 was found to sensitize cancer cells to chemotherapeutic agents, such as cisplatin [91]. In addition, inducible expression of R18 reduced tumorigenesis in mice bearing ZNF198-FGFR1–transformed hematopoietic cells [92]. Expression of difopein induced apoptosis in human glioma cells and suppressed tumor growth in mice [94]. These studies demonstrate the clinical importance of targeting the pro-survival function of 14-3-3 as a potential anticancer strategy.

In addition to R18 and difopein, well studied 14-3-3-recognition motif peptides (Mode I, II and III, see Obsil chapter) and other naturally occurring 14-3-3-binding peptides can also be utilized as antagonist peptides. Application of these peptides will lead to enhanced functional understanding of 14-3-3 biology. Importantly, these well-characterized peptides may also serve as templates for structure-based design of mimetics.

3.2.2. Small molecule 14-3-3 modulators

3.2.2.1. 14-3-3/client protein interaction inhibitors: Three avenues have been utilized for the discovery of small molecule 14-3-3 inhibitors, (i) computational-based virtual screening, (ii) chemical microarray-based screening with a peptide-small molecule hybrid library and (iii) bioassay-based high-throughput screening.

The crystallographic structure of 14-3-3 σ in complex with a nine residue peptide, MARSHpSYPA, was used as a starting point to design a pharmacophore for virtual screening [73]. Database searching with the peptide ligand pharmacophore model coupled with molecular docking with a series of scoring criteria led to the discovery of fourteen candidate compounds. BV02 (Fig. 1) was found to be a lead compound based on its bioactivity in cancer cells. This compound was specifically tested in cells with oncogenic Bcr-Abl-expression. The development of imatinib mesylate, which targets Bcr-Abl, has revolutionized cancer treatment due to its efficacy and safety profile. However, drug resistance has become a clinical problem for imatinib, due to a specific mutation in Bcr-Abl, T315I. BV02 appears to be active with an LD₅₀ of around 1 μ M in Ba/F3 cells expressing either WT or mutant Bcr-Abl, providing a potential lead for therapeutic development. In support of its role in blocking 14-3-3 activity, BV02 has been shown to release c-Abl from 14-3-3 and induce apoptosis in Bcr-Abl-expressing cells. Therefore, BV02 may be useful in Imatinib-resistant leukemia cells.

The second approach for the identification of small molecule 14-3-3 inhibitors was the use of small molecule microarray coupled with fragment-based synthesis [74]. Based on a defined 14-3-3-recognition motif sequence, RFRpSYPP, a peptide-small molecule hybrid library was created and used to screen for 14-3-3 binding molecules. From 293 compounds, five hits were shown to have desired affinity towards 14-3-3 σ . The five hits were used as fragments to generate six recombinant compounds. Compound 2–5 was found to exert the most potent inhibitory effect on 14-3-3 with an IC₅₀ of 2.6 μ M as measured by FP assay. A concentration of 100 μ M of compound 2–5 was used for confirming its ability to disrupt the 14-3-3 σ interaction with its binding partners in a GST-14-3-3 σ affinity pull-down assay and

to exert cellular effect in inducing markers of apoptosis in lung cancer cells in a caspase assay and cell cycle analysis assay. The phosphate of 2–5 appears to be critical for its inhibitory effect on 14-3-3 σ as shown by *in vitro* binding assay and cellular functional assays, as the non-phosphorylated counterpart is inactive.

The third approach for small molecule 14-3-3 inhibitor discovery is bioassay-based HTS and uHTS. Our lab has employed both 14-3-3 FP and TR-FRET assays in uHTS formats to screen diversity chemical libraries of over 200,000 compounds for new chemical scaffolds of 14-3-3 inhibitors. These uHTS campaigns have led to the identification of a series of small molecule 14-3-3 inhibitors, termed FOBISIN for FOurteen-three-three BInding Small molecule INhibitor [75](manuscript in preparation). The first of these FOBISIN molecules was a series of pridoxal-phosphate containing compounds, including FOBISIN 101 [75]. FOBISIN 101 was identified as a hit from the screening of the LOPAC library using a 14-3-3 γ /pS259-Raf-1 interaction-based FP assay. FOBISIN 101 was found to bind directly to 14-3-3 proteins, exhibiting a pan-14-3-3 inhibitor effect. It effectively disrupted 14-3-3/client protein associations with an IC₅₀ from 0.8 to 6.0 μ M for the interaction of full length Raf-1 with the seven isoforms of 14-3-3 in a GST-14-3-3 affinity pull-down assay. Functionally, FOBISIN 101 potently blocked 14-3-3 activation of ExoS enzymatic activity. Although at high concentrations (\sim 50 μ M), FOBISIN 101 inhibited colony formation of lung cancer cells (unpublished data), it remains to be determined whether this effect is due to the specific inhibition of 14-3-3 function. To understand the molecular basis of the effect of FOBISIN 101, the crystal structure of 14-3-3 ζ was solved in complex with FOBISIN 101. Results from these studies show a clear docking of FOBISIN 101 in the client binding site of 14-3-3 ζ , supporting a competitive inhibitor effect. Surprisingly, the double bond of FOBISIN 101, which links the pyridoxal-phosphate and benzoate moiety, was reduced by X-rays to create a covalent linkage of the pyridoxal-phosphate moiety to Lys120 in the binding groove of 14-3-3 ζ . These studies have not only revealed a novel class of 14-3-3 inhibitors, but also offer a potential pro-drug anticancer strategy, by which FOBISIN 101-like molecules are activated by radiation to specifically and potently inhibit 14-3-3 in radiation-treated tumor tissues. Preliminary structure-activity relationship studies have now identified additional compounds, with FOBISIN 106 exhibiting superior activity over that of FOBISIN 101 [75]. Further studies on the class of FOBISIN 101 molecules and other class of new chemical scaffolds currently under study are expected to lead to specific and bioactive small molecule 14-3-3 inhibitors.

3.2.2.2 14-3-3/client protein interaction stabilizers: Protein-protein interactions are dynamic processes, which require finely tuned association and dissociation mechanisms. Although disruption of 14-3-3/client protein interactions are expected to inhibit 14-3-3 function, constrained association may also impair the dynamic nature of the interaction networks, leading to 14-3-3 inhibition. While compounds in mammalian systems that stabilize 14-3-3/client protein interactions remain elusive, studies in plant systems have led to the discovery of two stabilizers of 14-3-3/client protein interaction: fusicoccin A and cotylenin A, two terpenoids isolated from fungus [95–96]. Under physiological conditions, plant 14-3-3 interacts with the C-terminal motif, YpTV, of plant plasma membrane H⁺ATPase in a highly regulated fashion [97]. Fusicoccin A secreted from a fungal plant pathogen can enhance the 14-3-3/H⁺ATPase interaction, leading to constitutive activation of the H⁺ATPase and the pathogenic effect. Structural analysis provided a clear explanation for this stabilizing effect, where fusicoccin occupied a space left between 14-3-3 and the C-terminal end of H⁺ATPase by binding with both ends of the proton pump and residues of 14-3-3. In this manner, fusicoccin A stabilizes the 14-3-3 protein complex and increases their affinity by about 100 fold. Cotylenin A assumes a similar role as fusicoccin A in stabilizing the 14-3-3/proton pump interaction.

Recent biochemical screening of a 37,000 compound library for 14-3-3/plant proton pump stabilizers led to the discovery of two structurally distinct compounds, pyrrolidone1 and epibestatin with K_d of 80 and 1.8 μM , respectively [98]. Structural analysis revealed the mechanism for their stabilization activity that is partially resembles the action of fusicoccin. It is remarkable that two structurally distinct compounds mimic the fusicoccin effect by stabilizing the 14-3-3/client protein interaction. These results indicate that bioactive small molecules can be developed to control 14-3-3 functions by stabilizing its interaction with specific client proteins.

4. Conclusions and future directions

14-3-3-mediated protein-protein interaction networks exert critical roles in the regulation of diverse cellular signaling pathways and have emerged as potential therapeutic targets in disease contexts such as cancer, neurological diseases, and infectious diseases. Due to the ubiquitous nature of 14-3-3 proteins, it is essential to identify dysregulated 14-3-3 proteins and/or dysregulated 14-3-3-mediated signaling networks that define potential therapeutic windows. Future studies are required to validate 14-3-3 targets in relevant diseases models with clinical correlations and also to define novel compounds that are potent and specific modulators of 14-3-3 proteins within these validated disease contexts.

14-3-3 inhibitor development has been pursued to study the biological functions of 14-3-3 proteins and for therapeutic intervention. For example, peptide inhibitors, such as R18 and difopein, have significantly advanced the 14-3-3 field. In addition, small molecule inhibitors of 14-3-3 protein-protein interactions are emerging (Fig. 1). Importantly, such small molecule 14-3-3 inhibitors allow the dynamic modulation of 14-3-3/client protein interactions. This ability to reversibly regulate 14-3-3/client protein interactions is expected to aid in our understanding of 14-3-3 biology and to lead to potential therapeutic discovery. However, the current generation of 14-3-3 inhibitor compounds still requires extensive characterization and significant improvement in order for them to become practical tools for the study of 14-3-3 biology. In particular, the cellular specificity and selectivity of these compounds for 14-3-3 remain to be established. As 14-3-3 proteins control diverse cellular pathways, development of compounds with enhanced isoform- or client-specific selectivity is highly desired. Due to the isoform-specific activity of 14-3-3 proteins, compounds that demonstrate a certain specific selectivity to a particular isoform may offer unique values. Furthermore, compounds that selectively interfere with the interaction of 14-3-3 with a critical target in a particular pathway will permit the dissection of the role of 14-3-3 in a pathway-specific manner. For the development of 14-3-3 inhibitors, effective biomarkers to validate 14-3-3-targeting effects need to be identified. While much optimization is still required to develop compounds that can be used as therapeutics, the currently available 14-3-3 inhibitor compounds offer invaluable starting points. In addition, stabilizers of 14-3-3 may have a potential role in 14-3-3 studies as well as in therapeutic development, for example as neuroprotective agents. Thus, a significant amount of work remains to identify the next generation of bioactive small molecule 14-3-3 modulators that exhibit desired specificity, selectivity and potency, which can be used for chemical biology and therapeutic discovery.

Highlight

14-3-3 proteins and 14-3-3-mediated protein-protein interaction networks are intimately involved in the regulation of a large number of physiological and pathological processes, which supports 14-3-3 as a novel class of potential therapeutic targets. Peptide and small molecule 14-3-3 modulators under development will offer new opportunities for further understanding of 14-3-3 biology and therapeutic discovery.

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Peptide antagonists

R18: PHCVPRDLSWLDLEANMCLP

Difopein: dimeric fourteen-three-three peptide inhibitor



Synthetic peptides and cell permeable fusions

Expression vectors and inducible expression vectors

Small molecule 14-3-3 modulators

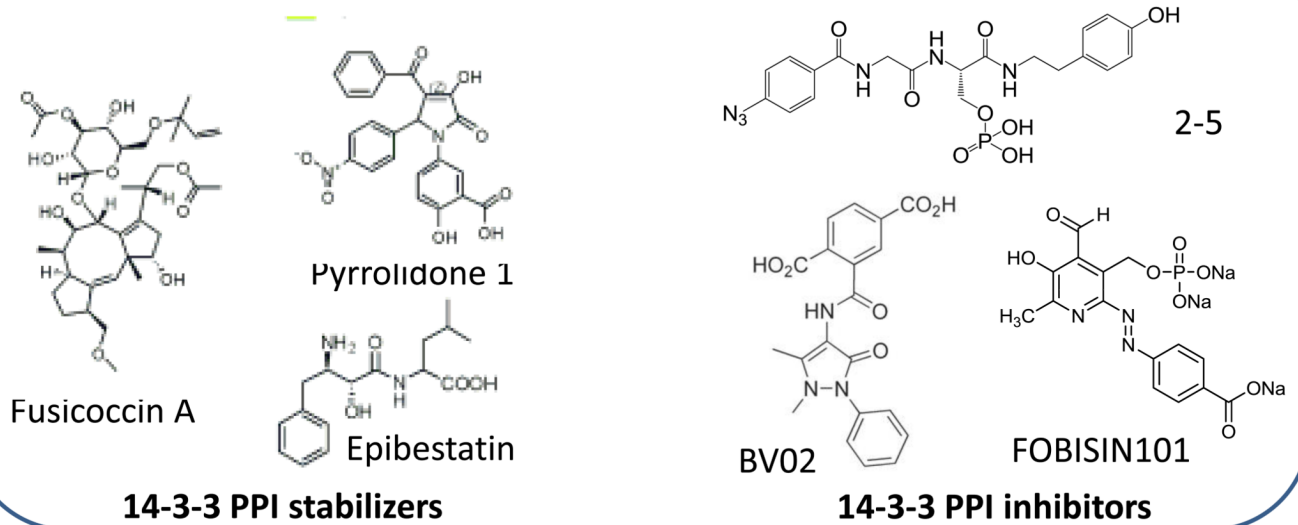


Fig. 1. Modulators of 14-3-3 protein-protein interactions

Upper panel: R18 peptide with key 14-3-3 binding residues in bold and difopein construct.

Lower panel: Two categories of small molecule 14-3-3 protein-protein interaction (PPI)

modulators: three PPI stabilizers (left) and three classes of PPI inhibitors.