Discovery and structural characterization of a small molecule 14-3-3 protein-protein interaction inhibitor

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The 14-3-3 family of phosphoserine/threonine-recognition proteins engage multiple nodes in signaling networks that control diverse physiological and pathophysiological functions and have emerged as promising therapeutic targets for such diseases as cancer and neurodegenerative disorders. Thus, small molecule modulators of 14-3-3 are much needed agents for chemical biology investigations and therapeutic development. To analyze 14-3-3 function and modulate its activity, we conducted a chemical screen and identified 4-(2Z)-2-[4-formyl-6-methyl-5-oxo-3-(phosphonatoxy)methyl]pyridin-2-ylidenel]hydrazinyl]benzoate, which we termed FOBISIN (Fourteen-three-three Binding Small molecule Inhibitor) 101. FOBISIN101 effectively blocked the binding of 14-3-3 with Raf-1 and proline-rich AKT substrate, 40 kD, and neutralized the ability of 14-3-3 to activate exoenzyme S ADP-ribosyltransferase. To provide a mechanistic basis for 14-3-3 inhibition, the crystal structure of 14-3-3ζ in complex with FOBISIN101 was solved. Unexpectedly, the double bond linking the pyridoxal-phosphate and benzoate moieties was reduced by X-rays to create a covalent linkage of the pyridoxal-phosphate moiety to lysine 120 in the binding groove of 14-3-3, leading to persistent 14-3-3 inactivation. We suggest that FOBISIN101-like molecules could be developed as an entirely unique class of 14-3-3 inhibitors, which may serve as radiation-triggered therapeutic agents for the treatment of 14-3-3-mediated diseases, such as cancer.

small molecule 14-3-3 modulator

Initially discovered as a protein abundant in the brain, the 14-3-3 family of proteins consists of seven defined isoforms (β, ε, γ, η, σ, τ, and ζ) in mammals and is widely expressed in all tissues and organs examined (1–3). 14-3-3 acts as an adaptor protein that controls the function of its target proteins through highly regulated protein–protein interactions. Studies on the interaction of 14-3-3 with phosphorylated Raf-1 led to the discovery of 14-3-3 as the founding member of the class of phosphoserine/threonine-binding protein modules (4–8). Reversible phosphorylation of target proteins at a defined motif dictates the 14-3-3 association in response to dynamic actions of cellular kinases and phosphatases. These 14-3-3 recognition motifs include the prototype sequence, RXxxS/TExP (mode 1), and RXxxS/TExP (mode 2), pSTXCOOH (mode 3), where x stands for any amino acid (8–11). The availability of well characterized 14-3-3 recognition motifs coupled with advanced genomics, proteomics, and functional biology approaches has revealed an entirely new landscape in which 14-3-3 binds a variety of signaling molecules, controlling their function in response to environmental signals (8, 9, 12–14). More than 200 ligand proteins have been identified for 14-3-3 (12). Depending on the nature of its target proteins, 14-3-3 binding impacts multiple signaling pathways that determine cell fate and organ development. For example, 14-3-3 association controls Raf signaling fidelity, neutralizes Bad-mediated apoptosis, and couples histone H3 with H4 to create a histone code for transcriptional elongation (2, 3, 15). Through these highly regulated interactions, 14-3-3 proteins govern diverse physiological processes as well as a wide range of pathophysiological events. For example, deregulated 14-3-3 signaling contributes to the development of a number of human diseases, such as cancer and neurodegenerative diseases (1–3). Thus, 14-3-3 proteins are promising molecular targets for probe discovery and therapeutic development.

In an effort to discover 14-3-3 protein–protein interaction modulators, we have previously reported the development and structural characterization of peptide 14-3-3 antagonists, R18 and difopein (16–18), which have been widely used in the field to manipulate 14-3-3/client protein interactions for functional studies. It is expected that small molecule 14-3-3 modulator discovery would provide added advantages to rapidly advance the 14-3-3 field, which impacts a wide range of biomedical areas. Here we report our experimental chemical screening effort, the identification and analysis of FOBISIN101 as a phosphoSer/Thr-mimetic agent, and the structural details of the FOBISIN101/14-3-3ζ interaction. This study revealed an unexpected covalent modification of 14-3-3ζ by a FOBISIN 101 derivative at a critical ligand binding site, Lys120, explaining its potent 14-3-3 inhibitory effect.

Results and Discussion

Using a fluorescence polarization-based 14-3-3 binding assay (19), we screened the LOPAC library for compounds that disrupt the interaction of 14-3-3ζ with the pS259-Raf-1 peptide and identified FOBISIN101 (F1 in Fig. 1A) as a potential 14-3-3 inhibitor (Fig. S1) (19, 20). F1 consists of a pyridoxal-phosphate moiety linked to p-amino-benzoate via an N = N bond. Because the screening assay utilized a 14-3-3-binding peptide, it is essential to demonstrate that FOBISIN101 is capable of disrupting the interaction of 14-3-3 with its full-length binding proteins. We employed three complementary biochemical and functional assays for this purpose. A Glutathione S-transferase (GST) fusion 14-3-3 affinity chromatography assay was used to examine the ability of F1 to disrupt the 14-3-3 association with two well-established partners, Raf-1 and proline-rich AKT substrate, 40 kD (PRAS40). The addition of increasing concentrations of F1 to the cell lysates led to a dose-dependent release of PRAS40 and Raf-1, supporting an effective inhibitory role of F1. This inhibi...
Ile217 (Fig. 2) the side chain of Lys49 and Asn173, while one face of the pyridine ring is orthogonal to the aromatic plane of each monomer. However, only the pyridoxal-phosphate moiety appears to influence 14-3-3 binding. 14-3-3 antagonists are known to affect protein–protein interactions, including those of Raf-1 and PRAS40 (Fig. 1A, Table S1). The crystal contains four monomers (two dimers) comprising 19% by weight of all seven 14-3-3 isoforms and their probable phosphate moieties. 14-3-3 bound to either the pS259-Raf-1 (PDB 3CU8) or pS10-histone H3 (25) (PDB 2CIN) peptide. In order to interact with phosphorylated ligands, 14-3-3 engages a cluster of basic or polar residues, including Arg56, Arg127, and Tyr128, which coordinates the binding of the phosphate group (Fig. 2E) and Asn173, whose side chain oxygen atom forms a hydrogen bond with Asp114, which is the Aβ1–Aβ4 interaction network forming a hydrogen bond with the main chain amide nitrogen of the residue C-terminal to the phosphoserine and involves an intramolecular interaction network forming a hydrogen bond with Asp114, which in turn forms a salt bridge with Lys120 (Fig. 2F). The conformation of peptide residues at +2 and beyond differs when the peptide exits from the 14-3-3 ligand binding groove, with Raf-1 to the left and histone H3 to the right of Lys49, as shown in Fig. 2E. It appears as though the phosphate group of covalently linked F1 shifted approximately 4 Å away from the phosphoserine binding site, towards Lys120 (Fig. 2G). Mutagenesis of 14-3-3ζ coupled with direct binding studies using isothermal titration calorimetry indicated the importance of R56 and R60 in the binding of native uncleaved F1 (Fig. S4, Table S2), which supports the proposed model in Fig. 2. We reasoned that the phosphate moiety of F1 might be critical for its inhibitory activity by mimicking the phosphorylated peptide motif for 14-3-3 binding. We thus generated the compound F2, which lacks the phosphate group, and observed that this compound had a drastically reduced effect in blocking 14-3-3 binding to Raf-1 or PRAS40 (Fig. 1B and C, Fig. S2) and in inhibiting 14-3-3-mediated activation of ExoS (Fig. 1F). Moreover, changes in the phenyl ring structure also showed some effect on the 14-3-3/Raf-1 interaction (Fig. S5), demonstrating the involvement of the azophenyl substructure in 14-3-3 interaction. These data support F1 as a 14-3-3 inhibitor and highlight its phosphate moiety as a primary functional component. We modeled intact F1 by superimposing the F1-bound structure of 14-3-3ζ to that of 14-3-3 bound to either the pS259-Raf-1 (PDB 3CU8) or pS10-histone H3 (25) (PDB 2CIN) peptide. In order to interact with phosphorylated ligands, 14-3-3ζ engages a cluster of basic or polar residues, including Arg56, Arg127, and Tyr128, which coordinates the binding of the phosphate group (Fig. 2E) and Asn173, whose side chain oxygen atom forms a hydrogen bond with Asp114, which in turn forms a salt bridge with Lys120 (Fig. 2F). The conformation of peptide residues at +2 and beyond differs when the peptide exits from the 14-3-3 ligand binding groove, with Raf-1 to the left and histone H3 to the right of Lys49, as shown in Fig. 2E. It appears as though the phosphate group of covalently linked F1 shifted approximately 4 Å away from the phosphoserine binding site, towards Lys120 (Fig. 2G). Mutagenesis of 14-3-3ζ coupled with direct binding studies using isothermal titration calorimetry indicated the importance of R56 and R60 in the binding of native uncleaved F1 (Fig. S4, Table S2), which supports the proposed model in Fig. 2. We reasoned that the phosphate moiety of F1 might be critical for its inhibitory activity by mimicking the phosphorylated peptide motif for 14-3-3 binding. We thus generated the compound F2, which lacks the phosphate group, and observed that this compound had a drastically reduced effect in blocking 14-3-3 binding to Raf-1 or PRAS40 (Fig. 1B and C, Fig. S2) and in inhibiting 14-3-3-mediated activation of ExoS (Fig. 1F). Moreover, changes in the phenyl ring structure also showed some effect on the 14-3-3/Raf-1 interaction (Fig. S5), demonstrating the involvement of the azophenyl substructure in 14-3-3 interaction. These data support F1 as a 14-3-3 inhibitor and highlight its phosphate moiety as a primary functional component. We modeled intact F1 by superimposing the F1 phosphate group onto that of phosphoserine and rotating the torsion angles to reach maximum overlap with the bound peptide (Fig. 3A). The model suggests that the side of the pyridoxal ring with the phosphate group superimposes well with peptide backbone...
could point to the peptide exit pathway similar to that of Raf-1 (Fig. 3, left box).

To explore the possible cause of the covalent modification of 14-3-3 by F1, we hypothesized that radiation exposure cleaves the N=N diazene bond thereby releasing the paraaminobenzoic acid moiety into the solvent, while the hydrogen binding interaction holds the pyridoxal-phosphate moiety in place within the 14-3-3 binding site (Fig. 3B). In this model, the reactive nitrogen of the pyridoxal-phosphate group is approximately 6–7 Å from either Lys120 or Lys49, respectively (Fig. 3C). However, the side chain Nϵ of Lys120 is roughly parallel, while that of Lys49 is roughly perpendicular, to the plane of the pyridoxal ring. We suggest that bond-breaking and bond-making processes proceed through specific attack trajectories. The preferred attack trajectory might be the one which lies parallel to the plane of the ring and facilitates the formation of a new nitrogen bond of the cleaved compound with the side chain of Lys120, leading to covalent modification and inactivation of 14-3-3 function. Indeed, mutating Lys120 to Glu alone is sufficient to inactivate 14-3-3ζ (Fig. S6).

The N=N bond in diazene compounds is generally sensitive to radiation and is known to undergo photolysis to generate reactive organic radicals (26). We note that another plausible source of the covalent adduct is through imine (Schiff base) formation between the ε-amino group of a lysine and the aldehyde group (with the loss of water) on the pyridoxal-phosphate portion of the inhibitor. However, three lines of evidence argue against this possibility. First, the electron density is of sufficient quality to identify the phosphate group, its associated pyridine ring, and the aldehyde (with a two bond length -C=O away from a ring atom). If the site of covalent attachment is via imine formation, irradiation-induced cleavage of the N=N bond would be reduced to NH2 (in this case, one bond length away from a ring atom). Second, the covalent adduct is only formed after exposure
to X-rays. It is not known whether imine formation with pyridoxal-phosphate requires photolysis. Gel digestion and mass spectrometric analysis of digested peptides obtained from F1-soaked crystals that were not exposed to X-rays showed no adduct peak (Fig. 3D, top box). Importantly, only the X-ray-treated samples showed a mass addition corresponding to a peptide fragment of residues 114-131 with modified Lys120 (Fig. 3D, bottom box). Therefore, the fragmentation and covalent adduct formation of F1 observed in the 14-3-3ζ crystal structure very well could be induced by X-ray radiation during data collection, as evident by the change of color (Fig. 2A). Third, assuming the site of covalent attachment is via imine formation and the pyridine ring takes on the same conformation (by flipping the ring 180° horizontally as shown in Fig. 2C), the paraaminobenzoic acid moiety would point to the solvent without any specific contact to the protein. However, the pyridoxal-phosphate (F3 in Fig. 1A) has much reduced potency (Fig. 1E), indicating that the phenyl ring structure still contributes to the inhibitory activity of F1, which is supported by data in Fig. S5.

In conclusion, we have identified and experimentally confirmed a series of small molecule phospho-binding site inhibitor and revealed structural details of such one molecule with 14-3-3ζ. Because of the specificity of the mode of binding as revealed by the cocrystal structural studies and the potent effect on both phosphorylated and nonphosphorylated client protein binding to 14-3-3 proteins, this pyridoxal-phosphate class of compounds are expected to define a unique class of 14-3-3 inhibitors for physiological and therapeutic investigations. It is important to note that the F1 class of compounds have been investigated as ioniotropic P2X receptor antagonists (20). Rich medicinal chemistry information around this structural scaffold will greatly facilitate their development as 14-3-3 modulators. Importantly, we also offer a prodrug concept for 14-3-3-mediated diseases. For example, F1-like molecules could be developed as radiation-triggered therapeutic agents for the treatment of cancer. It is envisioned that such 14-3-3 inhibitors alone may show negligible toxicity to the host; however, radiation therapy targeted to a particular tumor area may specifically cleave such designed 14-3-3 inhibitors and lead to their covalent modification and potent inactivation of 14-3-3 proteins in tumors.

Materials and Methods

Molecular and Cell Biology Reagents. The expression vectors for GST-14-3-3 and His-14-3-3 isoforms were constructed as previously described (27). Glutathione agarose beads and nickel-affinity columns were purchased from GE Healthcare. Anti-Raf-1 and anti-GST antibodies were from Santa Cruz and Glutathione agarose beads and nickel-affinity columns were purchased from Pierce Biotechnology. This assay monitors the interaction of recombinant GST-14-3-3 proteins with endogenous client proteins, such as PRAS40, in COS-7 cell lysate. Briefly, GST-14-3-3 protein (1 μM) immobilized on an anti-GST plate was incubated with a test compound before adding COS-7 cell lysates in 1% NP-40 lysis buffer (16). After incubation and washing, antibodies specific to PRAS40 along with peroxidase-labeled anti-rabbit IgG (50 μL; 1:1,000 dilution) were added. After washing, 100 μL of tetramethylbenzidine was added. The reaction was stopped with sulfuric acid (0.1 N) and recorded at 450 nm on an Envision™ reader (Perkin Elmer). IC50 values were calculated using GraphPad software.

GST Pull-Down Assay and Western Blotting. For binding assays, GST-14-3-3 proteins (1 μg) were preincubated with various concentrations of test compound before COS-7 cell lysates were added. Client proteins associated with GST-14-3-3 were captured by glutathione Sepharose beads while unbound proteins were removed by washing (1% NP-40 buffer). The fraction that was bound to the beads was analyzed by SDS-PAGE followed by immunoblotting with antibodies specific to Raf-1, PRAS40, and GST.

ExoS Activation Assay. To examine the functional effect of test compounds on 14-3-3 proteins, we utilized the 14-3-3-dependent ExoS ADP-ribosyltransferase assay (21). This assay is used as a functional readout for 14-3-3 inhibitors. Briefly, 14-3-3 protein was preincubated with test compounds, followed by incubation with ExoS in the presence of substrates (SBT, NAD, and 0.35 μCi of adenylate-3β-PNAD− as a reaction tracer). The reaction was terminated by spotting assay mixture onto P81 phosphocellulose paper (Whatman). After washing, radioactivity incorporated into SBT by ExoS was determined by liquid scintillation counting. Enzyme activities were expressed as picomoles of ADP-ribose incorporated per min per microgram of ExoS. The inhibitory effect of compounds was expressed as percent inhibition of ExoS activity over vehicle control.

Chemical Synthesis of Compound F2. A 5.1 mg sample of F1 was dissolved in 1.0 mL of hydrofluoric acid (48%, Sigma-Aldrich) and incubated in an ice bath for 2 h. The solution was adjusted to a pH of 5 using a saturated NaOH aqueous solution. The solution was dried under a vacuum using a rotary-evaporator at 30 °C. Precocool (~20 °C) ethanol was added to the remaining residue. The solid residue was filtered and the filtrate was dried to give 3.1 mg of a brown solid product. Yield: 89%.

ESI-NEG: [M-H] 314.1

H-NMR (400 MHz, CD3OD): 8.02–8.24 (4H, 6.30 (m, 1H), 5.35–5.38 (m, 2H), 2.63 (s, 3H).

Crystallography. The 14-3-3ζ protein (10 mg/mL in 20 mM Tris/HCl, pH 8.5, 100 mM NaCl) was crystallized by the hanging drop vapor diffusion method (Superdex 200 in a Pharmacia FPLC system). ExoS was purified as previously described (28). The LOPAC library was purchased from Sigma-Aldrich. COS-7 cells were grown in DMEM supplemented with 10% FBS.

Florencecence Polarization Assay and Chemical Screening. The 14-3-3 F3 assay was carried out in black 384-well microplates in a total volume of 50 μL (19). Assay reaction buffer (49 μL; 1 μM GST-14-3-3 and 2 mM TMR-p5259-Raf peptide in Heps buffer) was dispensed to each well. Test compound (1 μL of 2 mM stock in DMF) was added to the reaction buffer using a Scioclone liquid handler (Caliper LifeSciences). Plates were incubated at room temperature and the F3 value in millipolarization (mP) units was recorded with an Analyst HT reader (Molecular Devices). An excitation filter at 545 nm and an emission filter at 610 to 675 nm were used with a dichroic mirror at 565 nm. Data analysis was conducted using CambridgeSoft software. Compounds with recorded mP values less than three standard deviation from the negative controls were considered positive hits.

Enzyme-Linked Immunosorbtent Assay. The 14-3-3 ELISA assay was developed in 96-well microplates coated with either anti-GST antibody or glutathione (Pierce Biotechnology). This assay monitors the interaction of recombinant

GST-tagged 14-3-3 proteins with endogenous client proteins, such as PRAS40, in COS-7 cell lysate. Briefly, GST-14-3-3 protein (1 μM) immobilized on an anti-GST plate was incubated with a test compound before adding COS-7 cell lysates in 1% NP-40 lysis buffer (16). After incubation and washing, antibodies specific to PRAS40 along with peroxidase-labeled anti-rabbit IgG (50 μL; 1:1,000 dilution) were added. After washing, 100 μL of tetramethylbenzidine was added. The reaction was stopped with sulfuric acid (0.1 N) and recorded at 450 nm on an Envision™ reader (Perkin Elmer). IC50 values were calculated using GraphPad software.

Mass Spectrometry. Covalent adduct formation between 14-3-3ζ/K120 and fragmented F1 was verified by performing MALDI-TOF-MS analysis of V8-protease (New England BioLab) digested peptide fragments of the F1-protein crystals with or without X-ray exposure was added. The reaction was stopped with sulfuric acid (0.1 N) and recorded at 450 nm on an Envision™ reader (Perkin Elmer). IC50 values were calculated using GraphPad software.

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