VIP Protein–Protein Interactions

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Microarray-Assisted High-Throughput Identification of a Cell-Permeable Small-Molecule Binder of 14-3-3 Proteins**

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Protein–protein interactions (PPI) control important regulatory pathways in virtually all cellular processes.[1] For example, the phosphorylation-dependent PPI, tightly regulated by kinases, phosphatases, and a large number of proteins containing phosphopeptide-binding domains, forms the basis of the highly complex, cellular signal transduction network. Inappropriate interactions between proteins are known to cause many human diseases.[2] Inhibitors capable of disrupting protein–protein interactions, and especially those made of small, cell-permeable molecules, are therefore attractive leads for drug discovery.[3] In contrast to mainstream drugs, which are usually active-site inhibitors of enzymes (such as Gleevec for Abl kinase), and have historically been the focus of pharmaceutical research, the development of PPI inhibitors is perceived to be highly challenging, but at the same time rewarding, especially for treating diseases of less-common therapeutic targets, such as transcription factors. Examples include inhibitors that bind to different Src Homology 2 (SH2) domains, which have been extensively investigated.[4] SH2 domains are phosphotyrosine (*p*Tyr)-binding domains present in many important proteins (such as Src and Abl kinases). Phosphoserine/phosphothreonine (*p*S/*p*T)-binding domains constitute another major class of signaling domains with prominent roles in regulation of mitosis, DNA damage, and apoptosis.[5] Among them, 14-3-3 proteins, made up of a family of acidic, dimeric, α-helical, cup-shaped molecules present in all eukaryotic cells, have been well-characterized.[6] Small-molecule-based PPI inhibitors of 14-3-3 proteins, however, are not currently available.

In humans, seven highly homologous 14-3-3 isoforms are present (β, ε, η, γ, σ, τ, and ζ). Together, they regulate several hundred proteins, many of which are important pharmaceutical targets, such as Raf, p53, Cdc25, and histone deacetylases (HDACs).[6a] To date, only 14-3-3σ, through its direct interaction with p53 (a major tumor suppressor protein), has been linked to cancer. Inactivation of 14-3-3σ has been shown to be crucial in tumorigenesis. Due to the highly conserved nature of residues in 14-3-3 isoforms that constitute the *p*S/*p*T peptide-binding pocket, most 14-3-3 family members display significant functional redundancy both in vitro and in vivo.[6b,c] The elegant work of Yaffe et al. has unequivocally established that most proteins bind to 14-3-3 proteins through a consensus hexa- or heptaphosphopeptide binding motif, RXX[*p*S/*p*T]XP or RXXX[*p*S/*p*T]XP, respectively (where X represents any amino acid, but is in most cases an aromatic/hydrophobic residue).[6b] Recent studies have further identified sequences such as RFR*p*SYPP and RLSH*p*SLPG as optimal peptides recognized by all 14-3-3 proteins,[6b] as well as LFG*p*SLLR and LFG*p*SLVR that confer some preference toward the 14-3-3σ over other isoforms.[7]

The high redundancy of 14-3-3 proteins makes classical gene knockout approaches ill-suited for the study of their general functions in cells, as single gene knockout will be compensated for by other 14-3-3 isoforms, and simultaneous knockout of all seven genes would be technically impossible. Fu et al. used the so-called difopein (two 18-amino acid peptides joined by a linker) as a general 14-3-3 PPI inhibitor. Upon overexpression in mammalian cells from the corresponding DNA, the compound was shown to disrupt 14-3-3/ligand interaction, resulting in cell cycle arrest and apoptosis.[8a,b] This method, however, depends on transient transfection and is difficult to control (in terms of time, concentration, etc). Yaffe et al. recently showed the use of caged phosphopeptides for both in vitro and in-cell studies of 14-3-3 functions in a temporally controlled manner.[8c] The peptides were chemically synthesized and conjugated (to a cell-penetrating peptide, or CPP) prior to being delivered into cells, and subsequently "turned on" by UV irradiation. Although elegant, this approach lacks a "turn-off" mechanism and requires the use of UV, which is harmful to cells. Furthermore, these peptides lack sufficient cellular permeability (thus the need for CPP conjugation) and hydrolytic stability (they are readily degraded by endogenous proteases in cells), making them unsuitable for general chemical biology applications. Our ongoing interests in 14-3-3 proteins and their protein–protein interacting partners as potential therapeutic targets have prompted us to ask whether it is possible to develop cell-permeable small molecules that are capable of binding to all 14-3-3 proteins, or to a specific isoform, namely 14-3-3σ. We envisioned that such molecules would be highly valuable not only as a research tool for studies of 14-3-3 biology (especially that of 14-3-3/ligand interaction), but also as potential anti-cancer agents. Herein, we present one such compound, **2-5** (Figure 1). To the best of our knowledge, this compound is the first small-molecule PPI inhibitor of 14-3-3 proteins. We have carried out extensive biochemical assays to confirm that **2-5** directly competes for 14-3-3/ligand binding in a potent and

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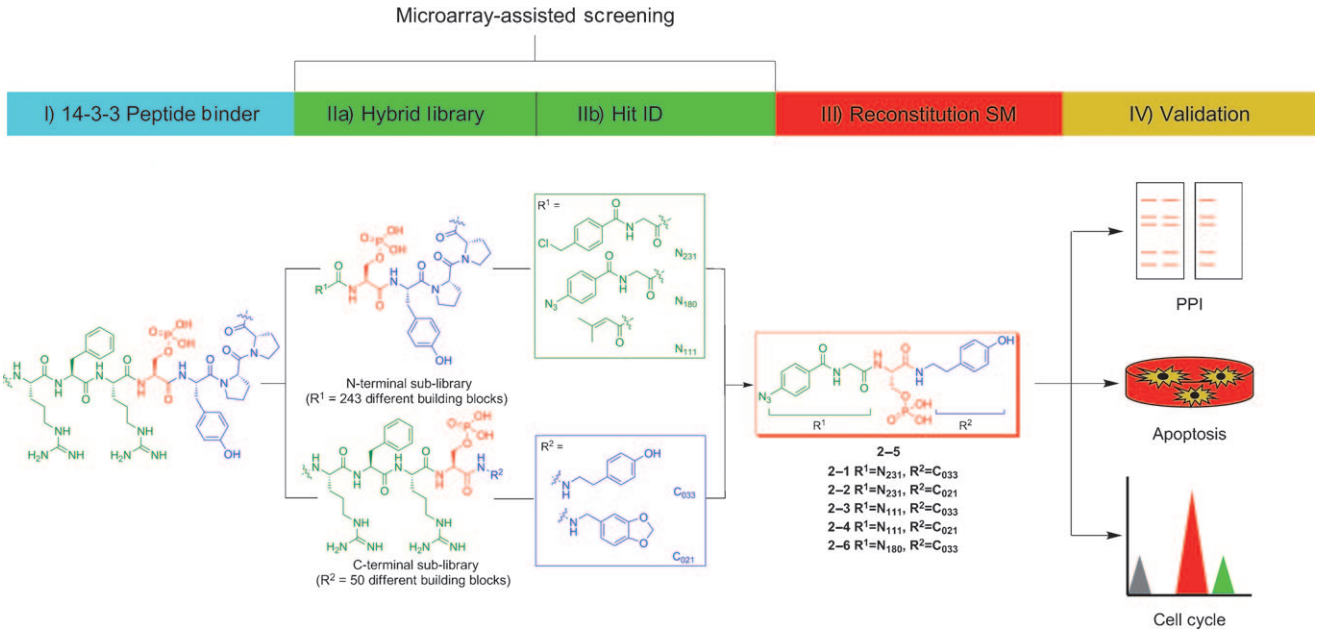
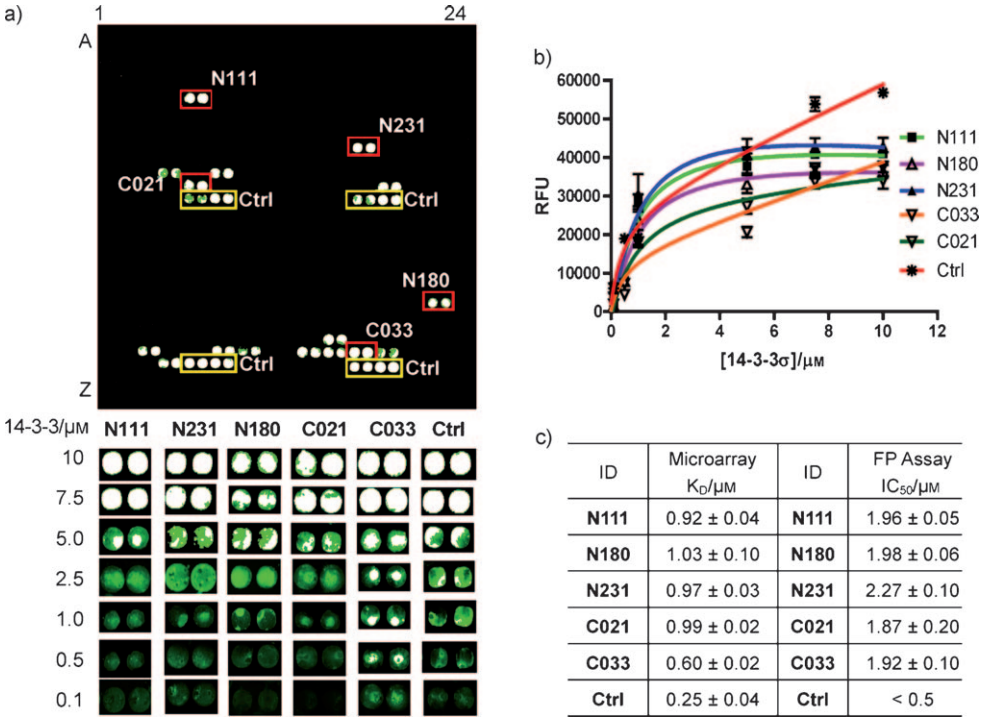


Figure 1. Overview of small-molecule microarray-assisted, fragment-based high-throughput identification of potential peptide–small-molecule hybrid molecules (steps I and II), which were subsequently reconstituted (step III) to obtain the corresponding cell-permeable, small-molecule hits, followed by biological validation (step IV). ID = identification, SM = small molecule, PPI = protein–protein interaction.

specific manner in vitro. In mammalian cells, it induces apoptosis and cell cycle arrest.

To identify **2-5** and related compounds in high throughput, we used the recently developed small-molecule microarray (SMM) technique,[9] and followed the workflow shown in Figure 1. To convert the original optimal 14-3-3-binding peptide, RFR*p*SYPP, into a small-molecule-based 14-3-3 binder, we first synthesized a peptide-small molecule hybrid library (step I); it is made up of the 243-member N-terminal and 50-member C-terminal sub-libraries (for synthesis, see the Supporting Information, Scheme S1). We retained the key *p*S residue in the library, and at the same time systematically replaced the two flanking peptide fragments (RFR and YPP) with commercially available acid and amine building blocks (R^1 and R^2, respectively, in Figure 1, corresponding to the $P_{-1/-2}$ and $P_{+1/+2}$ positions in the original peptide). A recently introduced library design concept termed "fragment-based combinatorial chemistry" was adopted to ensure that a sufficient chemical space was covered by a limited number of building blocks (243 R^1 and 50 R^2) , and at the same time all library members retained some degrees of binding affinity toward 14-3-3 proteins.[7] Aliphatic/aromatic building blocks were chosen in the library construction, as they may improve the cell permeability of potential "hits", and based on X-ray structures of 14-3-3 proteins, the binding pockets immediately adjacent to the *p*S-binding site are mostly hydrophobic.[6b,c] Briefly, the N-terminal sub-library was made by coupling 243 different acids (R^1) to the rink amide resin pre-loaded with peptide NH_2-*p*SYPP (where R^1 is an aliphatic building block) or NH_2-G*p*SYPP (where R^1 is an aromatic building block). Similarly, the C-terminal sub-library was synthesized by initial immobilization of 50 different amines (R^2) onto the PL-FMP resin by reductive amination (Supporting Information, Scheme S1), following previously published procedures,[10] then coupling of the peptide sequence RFR*p*S. For both sub-libraries, biotin and a GG linker were introduced at either the N- or C-terminus of each member to facilitate subsequent immobilization onto avidin-functionalized glass slides. All the compounds were characterized by liquid chromatography–mass spectrometry and most were shown to be of the correct molecular weight and sufficient purity for direct microarray applications.[11]

The entire library, together with a positive control peptide, Biotin-GG-RLSH*p*SLPG, was spotted (in duplicate) to generate the corresponding SMM. To ensure uniform immobilization, spotted slides were stained with Pro-Q diamond dye (Supporting Information, Figure S1); minimal slide-to-slide and spot-to-spot variations ($r > 0.95$) were observed. We then examined whether this fragment-based, peptide–small-molecule hybrid SMM could be used to identify, in high throughput, ligands that bind strongly to 14-3-3 proteins (step II in Figure 1). A fluorescently labeled, recombinant GST-14-3-3σ was used in the SMM screening (Figure 2); upon data analysis, highly reproducible (as judged from duplicated spots/slides), concentration-dependent binding profiles were observed for five of the 293 compounds (boxed in red in Figure 2a). The positive control peptide (boxed in yellow in Figure 2a) also showed a robust binding profile. Microarray-based K_D determination was subsequently carried out (Figure 2b); besides the control peptide, which showed an apparent K_D of 0.25 μM (compared to the reported value of about 200 nM[7]), the newly identified ligands (N111, N180, and N231 from the N-terminal sub-library, and C021 and C033 from the C-terminal sub-library) all showed relatively good affinity toward 14-3-3σ (0.6–1.03 μM in K_D). Further validation was carried out with a



c)

ID	Microarray K_D/μM	ID	FP Assay IC_{50}/μM
N111	0.92 ± 0.04	**N111**	1.96 ± 0.05
N180	1.03 ± 0.10	**N180**	1.98 ± 0.06
N231	0.97 ± 0.03	**N231**	2.27 ± 0.10
C021	0.99 ± 0.02	**C021**	1.87 ± 0.20
C033	0.60 ± 0.02	**C033**	1.92 ± 0.10
Ctrl	0.25 ± 0.04	**Ctrl**	< 0.5

Figure 2. SMM-assisted identification of potential peptide–small-molecule hybride binders of 14-3-3, and quantitative K_D determination. a) Microarray image showing binding profiles of the 293-member library against fluorescently labeled GST-14-3-3σ. Spots corresponding to the control peptide RLSH*p*SLPG and positive "hits" are boxed in yellow and red, respectively. Other unboxed spots were weak binders (see Supporting Information, Figure S2) that were rejected without further follow-ups. b) Microarray-based K_D determination of the control peptide and five "hits". The corresponding dose-dependent microarray images, shown below Figure 2a, were obtained by screening the SMM with varied concentrations of the fluorescently labeled 14-3-3σ (0.1 to 10 μM). RFU = relative fluorescence unit. (c) Summary of the microarray-measured K_D and the corresponding IC_{50} values obtained from competitive fluorescence polarization (FP) experiments.

competitive fluorescence polarization (FP) assay (Figure 2c);[12] the same set of compounds emerged as "hits" and with IC_{50} values within the same range as the microarray-measured K_D, indicating that are all likely to be true binders of 14-3-3σ, and the corresponding R^1 and R^2 building blocks (boxed in green and blue, respectively, in Figure 1) may be further reconstituted to obtain the corresponding small molecule-based 14-3-3 PPI inhibitors (step III in Figure 1).

From the above peptide–small-molecule hybrid library, we have rapidly identified key building blocks (i.e. three R^1 and two R^2) capable of substituting peptidyl recognition fragments in a known 14-3-3-binding phosphopeptide without significant loss of its original binding property. We then reconstituted the corresponding phosphoserine-containing small molecules (**2-1** to **2-6** in Figure 1; step III). All six potential 14-3-3 small molecule binders (from three R^1 and two R^2) were synthesized. The non-phosphorylated controls, **1-1** to **1-6**, were prepared in a similar fashion (Supporting Information, Scheme S2).

Competitive FP experiments showed potent dose-dependent binding of **2-1** to **2-6**, but not the non-phosphorylated counterparts (**1-1** to **1-6**), toward 14-3-3σ, giving rise to the corresponding IC_{50} values of 2.6–3.6 μM, which are only two- to threefold less potent than the parental peptide–small-molecule hybrids (Figure 3a). It should be noted that, unlike most active site-targeted enzyme inhibitors (which are often nanomolar or even picomolar inhibitors), small-molecule micromolar PPI inhibitors are considered reasonably potent inhibitors.[3] In our case, the most potent inhibitor, **2-5**, with a IC_{50} value of 2.6 ± 0.4 μM, is only about tenfold lower in binding affinity than its peptide counterpart,[6,7] thus making it a good candidate as a potential cell-permeable, small-molecule 14-3-3 PPI inhibitor. This compound was therefore chosen for all subsequent studies.

To investigate whether compound **2-5** competes with cellular proteins for 14-3-3 binding in a biological complex, pull-down experiments were performed with A549 cell lysates.[8c] Bead-immobilized GST-14-3-3σ was pre-incubated with **2-5** (**1-5** and DMSO were used as negative controls) and then added to cell lysates. 14-3-3-bound proteins were separated by SDS-PAGE followed by immunoblotting with a group of different antibodies that recognize either the 14-3-3 phosphopeptide-binding motif, p53, or Raf-1 substrate proteins. As shown in Figure 3b, the phosphoserine-containing small molecule **2-5** was able to completely disrupt PPI between 14-3-3σ and its substrate proteins. On the other hand, a same amount of the non-phosphorylated control **1-5** was unable to disrupt the binding between 14-3-3σ and its endogenous substrates. This result indicates that **2-5** is a good small-molecule PPI inhibitor of 14-3-3 proteins in vitro. The cell permeability and in-cell biological activities of **2-5** were subsequently assessed. A standard cell permeability assay using MDCK cells indicated **2-5** indeed possesses reasonable cell permeability with a P_{app} value of 554 nm s^{-1}. Previously, it had been shown that expression of 14-3-3/ligand interaction inhibitors (namely difopein) in mammalian cells leads to induction of apoptosis and cell death. We therefore tested the cytotoxicity of **2-5** in A549 cells using the XTT cell proliferation assay (Figure 3c); results indicate that, at a concentration of 100 μM, **2-5** caused a large amount of cell death starting from 4 h to up to 12 h of drug treatment. The negative control (**1-5**) on the other hand had a significantly lower but noticeable degree of cell toxicity, indicating that although both compounds might have some intrinsic cytotoxicity, phosphorylation of **1-5** (that is, giving **2-5**) had drastically improved its cell-killing activity, presumably through the

a)

ID	FP Assay IC_{50}/μM	ID	FP Assay IC_{50}/μM
1–1	N.A.	**2–1**	3.2 ± 0.4
1–2	N.A.	**2–2**	3.3 ± 1.0
1–3	N.A.	**2–3**	3.6 ± 0.1
1–4	N.A.	**2–4**	3.1 ± 0.2
1–5	N.A.	**2–5**	2.6 ± 0.4
1–6	N.A.	**2–6**	3.6 ± 0.7

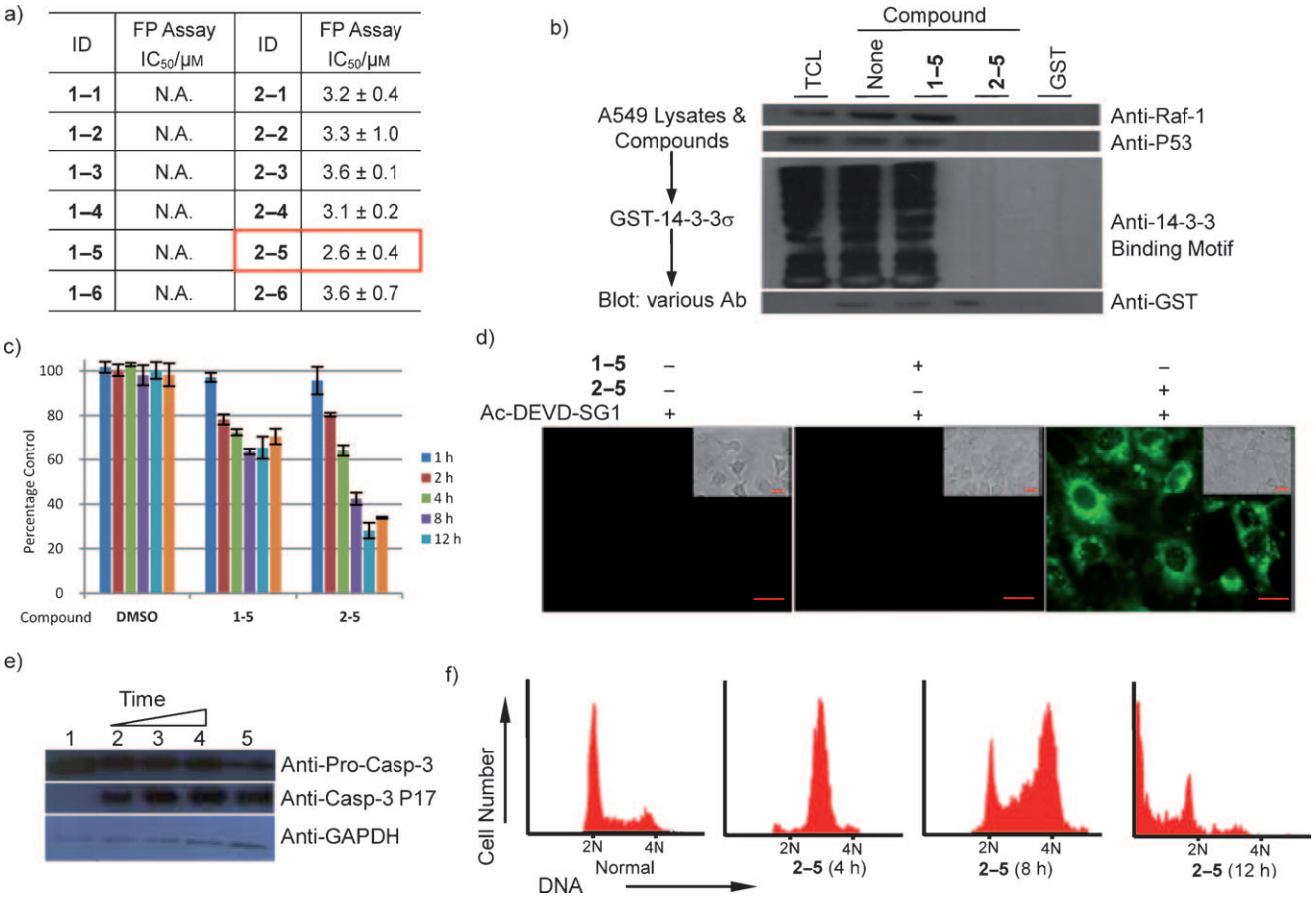


Figure 3. a) Summary of IC_{50} obtained from competitive FP experiments for **2-1** to **2-6** and their non-phosphorylated controls **1-1** to **1-6**. (N.A. = IC_{50} too high to be determined within experimental settings). (b) In vitro competitive binding of **2-5** with cellular proteins for 14-3-3σ binding. 100 μM of compounds **1-5** and **2-5** were pre-incubated with bead-immobilized GST-14-3-3σ then added to A549 cell lysates. Bound proteins were detected by SDS-PAGE and immunoblotting with antibodies against various 14-3-3-binding partners. Controls show proteins bound by GST-14-3-3σ beads in the absence of compounds, or by beads alone. TCL denotes total cell lysate; GST denotes beads alone. c) Time-dependent (1–24 h) inhibition of A549 cell proliferation by **2-5** (**1-5** as control) using XTT assay. Data represent the average standard deviation for two independent trials. d) Fluorescence microscopic images of A549 cells treated with compound **2-5** or **1-5** (100 μM), respectively. Caspase activity was monitored by addition of Ac-DEVD-SG1 as previously reported.[13] All images were acquired in the same way. Insets: DIC images. Scale bars = 10 μm. e) Immunoblotting results of the cells from (d), detected with anti-procaspase-3 and anti-caspase-3 (P17) antibodies. Lane 1: uninduced A549 lysate; lanes 2–4: **2-5** treated with A549 for 2, 8, and 12 h, respectively; lane 5: STS-treated cells (12 h). f) Flow cytometry results of A549 cells treated with 100 μM of **2-5** for 4, 8, and 12 h (Normal: no **2-5**).

increase in its binding affinity toward 14-3-3 proteins. We then investigated whether **2-5**-induced cell death was a direct result of caspase activation and apoptosis, as previously observed for other 14-3-3/ligand interaction inhibitors.[8a,b] A549 cells treated with **2-5** were monitored in a live-cell bioimaging experiment using the Ac-DEVD-SG1 fluorogenic caspase 3/7 substrate.[13] As shown in Figure 3d, A549 cells treated with **2-5** and staurosporine (STS; a positive control; data not shown) developed a strong green fluorescence, indicating apoptosis had occurred. In contrast, no significant increase in fluorescence was observed for nonapoptotic cells, even after extended incubation. Addition of a caspase 3/7 inhibitor, Ac-DEVD-CHO, to the STS and **2-5**-treated cells rendered the fluorescence undetectable (see Supporting Information). Further analysis of the corresponding cell lysates by SDS-PAGE and immunoblotting experiments unambiguously confirmed the time-dependent activation of caspase 3 activity in both the STS and **2-5** treated cells (Figure 3e). These results agree well with previous studies that disruption of 14-3-3/ligand interactions leads to caspase-mediated apoptosis,[8a,b] and further supports the hypothesis that 14-3-3/ligand interactions are required for critical cellular processes, including regulating pro-survival cell signaling.[6] Finally, 14-3-3/ligand interaction inhibitors are known to regulate cell cycle by causing premature cell cycle entry, release of G1 cells from interphase arrest, and loss of the S-phase checkpoint after DNA damage.[8c] We wondered if **2-5** behaves similarly in a flow cytometry-based cell-cycle experiment (Figure 3f); by measuring the population of cells containing cleaved DNA upon treatment with **2-5**, we observed a dramatic increase in the fraction of sub-G1 cells, indicating internucleosomal DNA cleavage (which is a commonly used marker of apoptosis). **1-5**-treated cells, on the other hand, behaved in a similar fashion to normal cells, which again validates the critical roles that 14-3-3 proteins play in cell cycle regulation.[8] Lastly, molecular docking

studies were carried out to explore the binding mode of **2-5** with 14-3-3σ, and results showed that, as might be expected, that **2-5** binds 14-3-3σ well in the phosphopeptide binding site of the protein (Supporting Information, Figure S5).

In conclusion, we have successfully demonstrated that the SMM technique offers a highly miniaturized yet powerful approach for rapid identification of small-molecule PPI inhibitors. From this study, through the identification of a cell-permeable small-molecule PPI inhibitor that targets 14-3-3 proteins, we have successfully developed a versatile chemical biology approach for studies of 14-3-3 biology. Compared to other existing methods,[8] our strategy makes use of the newly discovered compound **2-5**, which can be administered to cells by conveniently adding it to the cell medium. The method therefore does not require ectopic delivery of genetic materials or asynchronously delivered peptides/proteins. It also possesses other reasonable "drug-like" properties, such as small size and hydrolytic stability (see Supporting Information, Figure S4).[14] All of these attributes should make **2-5** a valuable chemical for probing 14-3-3 biology. We note that in a recent work, Waldmann, Ottmann and co-workers have identified small molecules that selectively stabilize, rather than disrupt, 14-3-3/ligand interactions.[15] We believe these two types of reagents might offer a powerful synergistic combination for future studies of 14-3-3/ligand interactions and associated signaling pathways. Our strategy should be directly applicable to other signaling modules that are functionally redundant, in which the protein of interest is part of a large conserved protein family, including other modular protein domains that recognize phosphoamino acids.[4,5] Throughout the course of our study, we have further expanded the utility of SMM beyond some of its most recent applications.[9,11,16]

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