Supporting Information

Virtual Screening and Experimental Validation Reveal Novel Small-Molecule Inhibitors of 14-3-3 Protein-Protein Interactions

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Computer Aided Molecular Modelling

Analysis of 14-3-3 Complex Structures

The virtual screening workflow is focused on our hypothesis that the phosphate group of pSer or pThr has the strongest pharmacophoric property of 14-3-3-binding proteins. This was elucidated by an analysis of crystal structures of 14-3-3 proteins bound to their phosphorylated partners. We analyzed nine randomly chosen 14-3-3 complex structures from the Protein Data Bank (PDB)^[1] with a resolution ≤ 2.5 Å.^[2–9] The main chains of the 14-3-3 proteins were aligned using the Molecular Operating Environment (MOE).^[10] Subsequent analysis of the passively superimposed phosphopeptides showed that the relative orientation of the phosphorylated residue and especially that of the phosphate group with an average RMSD of 0.51 Å itself is highly invariant (Figure 1). The phosphate group is coordinated via five conserved hydrogen bonds to the side chains of R56, R129, Y130 and in some cases to K49.

Virtual Screening Procedure

We used a generalized phosphonate as substructure (SMARTS pattern: [C,O][P]([OH])(=[O])([OH]) for the initial ligand-based virtual screening. The entire ligand-based screening is implemented as a single workflow in Pipeline Pilot (PP).^[11] Using the described substructure we first filtered the *all now* subset of the ZINC database (release 11; 8,061,769 compounds) which reduced the library to 2,349 compounds.^[12] The second step included a *Lipinski filter* for 'Rule of Five' compliance and an *HTS filter* to ensure druglikeness of the selected compounds. Both filters are components of the chemistry package of PP. This step reduced the library to 1,502 compounds. To get rid of multiple phosphorylated molecules and to gain some internal rigidity the last filter step only let pass compounds with a single query substructure match and at least one ring system ending up with 1,012 compounds. A visual inspection of this selection indicated on the presence of several related compound clusters. Therefore we calculated a diverse set of compounds based on 2D extended-connectivity fingerprints with a desired number of 500 compounds in the final selection.^[13] For this purpose the *Diverse Molecules* component of PPs data modeling package was applied finally yielding 512 compounds. This selection was further evaluated by a structure-based approach with ligand-receptor docking. For this purpose we employed software tools from the Molecular Modeling Platform of Schrödinger.^[14] To create a receptor grid we used an in-house crystal structure of 14-3-3\sigma in complex with a Mode-III phosphopeptide (PDB ID: 3P1N) with a resolution of 1.39 Å.^[15] The receptor was prepared using the *Protein Preparation Wizard* with slightly modified settings. We kept

the peptide and surrounding waters for optimization of the hydrogen bond network and deleted them afterwards. The compound selection was prepared with *LigPrep*.^[16] The docking was performed with *Glide* in XP mode.^[17–19] We visually inspected the 200 top-ranked docking poses according to their docking score and selected 14 compounds for experimental validation. The primary selection criterion was the placement of the phosphonate part of the docked compounds in order to satisfy our pharmacophore hypothesis.

General Procedures and Materials

His-14-3-3 ξ and 14-3-3 σ - Δ C were expressed and purified as described before.^[20] The p53_{385-393-pT387}-peptide (FK-pT-EGPDSD) and the FAM-labeled C-Raf_{252-264-pS259}-peptide (FAM-SQRQRST-pS-TPNVH-OH) were obtained from Biosyntan, Berlin, Germany. The biotinylated Raf_{251-280-p5259}-peptide (H-LSQRQRST-pS-TPNVHMVSTTLPVDSRMIEDAK(Biotin)-OH) was synthesized by GenScript, Piscataway, USA. The HTRF reagents (anti-His-d2 and Streptavidin-Tb) were supplied by Cisbio, Berlin. All other reagents and buffer components were purchased from commercial suppliers in analytical grade and used without further purification. Non-linear regression was performed using GraphPad Prism (version 5.03), GraphPad Software, San Diego California, USA. All compounds were purchased from InterBioScreen. The compound identifiers are STOCK1N-27073 (A13), STOCK1N-29704 (A3), STOCK6S-19901 (A4), STOCK6S-20951 (A11), STOCK6S-21429 (B2), STOCK6S-22115 (A5), STOCK6S-23126 (A1), STOCK6S-23301 (B13), STOCK6S-23618 (A2), STOCK6S-23822 (B20), STOCK6S-23881 (B21), STOCK6S-24610 (B22), STOCK6S-25642 (B11), STOCK6S-25681 (B14), STOCK6S-26034 (B7), STOCK6S-26036 (A6), STOCK6S-26169 (B10), STOCK6S-26928 (B23), STOCK6S-27243 (A10), STOCK6S-28426 (B25), STOCK6S-29765 (B26), STOCK6S-29992 (B27), STOCK6S-30099 (B6), STOCK6S-30286 (B12), STOCK6S-30315 (A9), STOCK6S-30541 (B1), STOCK6S-30569 (B28), STOCK6S-30980 (A12), STOCK6S-31316 (B36), STOCK6S-31619 (B3), STOCK6S-31676 (B4), STOCK6S-32535 (B29), STOCK6S-33871 (B16), STOCK6S-34461 (B30), STOCK6S-35347 (A14), STOCK6S-35412 (B9), STOCK6S-36239 (B32), STOCK6S-37402 (B15), STOCK6S-37412 (B37), STOCK6S-37663 (B33), STOCK6S-39246 (A8), STOCK6S-39499 (B17), STOCK6S-39700 (B34), STOCK6S-39852 (A7), STOCK6S-43021 (B35). Compounds were dissolved and stored as 20 mM stock solution in DMSO.

Fluorescence Polarization and HTRF Assay Procedures

Analysis of Tracer Peptide

From a 50 μ M stock solution in water the fluorescently labeled peptide was diluted to 100 nM in 1 x HBS containing 0.1 % Tween, 0.05 % BSA. This solution was used to prepare a serial dilution series of His-14-3-3ζ (from a Stock solution of 1.5 mM) in a 384 microwell plate (Greiner 781900) starting with a concentration of 150 μ M in well 1. After 1 h incubation, the plate was analysed using a Tecan Infinite F500 microplate reader and standard settings for fluorescein anisotropy (λ_{ex} : 485 nm, λ_{em} : 535 nm, Integration-Time: 50 μ s). The resulting anisotropy values were plotted against the protein concentration and the resulting curve was fitted against a single site binding model without correcting for receptor depletion:

$$y = r_{free} + \frac{\left(r_{bound} - r_{free}\right) \times X}{K_D + X}$$
(1)

Initial Testing of Selected Compounds from Virtual Screening

From a 50 μ M stock solution in water FAM-Raf_{252-264-p5259}-peptide was diluted to 100 nM in 1 x HBS containing 0.05 %Tween, 0.05 % BSA. To this solution was added His-14-3-3ζ (from a stock solution of 1.5 mM) to reach a final concentration of 20 μ M. The resulting solution was incubated with 250 μ M of the compound (from a stock solution in DMSO) in question in a 384 microwell plate (Greiner 781900). After 1 h incubation, the plate was analyzed using a Tecan Infinite F500 microplate reader and standard settings for fluorescein anisotropy (λ_{ex} : 485 nm, λ_{em} : 535 nm, Integration-Time: 50 μ s). The resulting anisotropy values were converted into %-inhibition and compounds with inhibition >50 % were selected for further evaluation.

Determination of IC₅₀ Values Using the Fluorescence Polarization Assay

Compounds were analyzed in triplicates applying the protocol described above using the FAM-Raf_{252-264-p5259}-peptide as fluorescent tracer and a serial dilution starting at 1 mM concentration. Resulting anisotropy values were normalized and fitted against a three parameter logistic fixed bottom model (Equation 2) to yield IC_{50} values.

$$y = \frac{top}{1 + 10^{(x - \log(IC50))}}$$
(2)

Determination of IC₅₀ Values Using the HTRF Assay

For the HTRF assay the biotinylated Raf_{251-280-p5259}-peptide was diluted to 100 nM (from a stock solution of 200 μ M in Water) in 1 x HBS containing 0.05 % Tween, 0.1 % BSA. To this solution was added His-14-3-3ζ (from a Stock solution of 1.5 mM) to reach a final concentration of 200 nM. This solution was used to prepare a serial dilution series of the compounds to be tested starting from 500 μ M in a 384 microwell plate (Greiner 781900). Afterwards 10 μ L of the HTRF-Stock solution (3200 μ L assay-buffer, 20 μ L Streptavidin-Tb, 20 μ L anti-6His-d2; HTRF-reagent stock solutions prepared according to the manufacturers description) was added to each well. After incubation at room temperature for 30 min the plate was analyzed using an Tecan Infinite F500 microplate reader and standard settings for HTRF (Wavelength 1 (Tb): λ_{ex} : 340 nm, λ_{em} : 620 nm, Wavelength 2 (d2): λ_{ex} : 340 nm, λ_{em} : 665 nm, Lag-Time: 50 μ s, Integration-Time: 100 μ s). The d2/Tb-ratio was normalized and plotted against logarithmic compound concentration. For the calculation of IC₅₀ values the curve was fitted against a three parameter logistic fixed bottom (Equation 2) model to yield IC₅₀ values.

MMP-1 mRNA Expression Assay

Cell Culture

The human fetal lung fibroblast cell line IMR-90 (ATCC: CCL-186) was cultured in Dulbeccos's Minimal Essential Medium (DMEM, PAN BIOTECH GmbH) additionally containing 4.5 g/l glucose, sodium pyruvate, 2 mM L-glutamine, 1 % non-essential amino acids, 3.7 g/l NaHCO₃ and 20 % heat inactivated fetal calf serum in a humidified 37°C incubator and 5 % CO₂. MMP-1 expression was induced by treating 80-90 % confluent IMR-90 cells using 15 μ g/ml recombinant 14-3-3 σ for 24 h.^[21]

RNA Extractions and RT-qPCR

Total RNA was purified using the TRIsure method (Bioline: BIO-38032) following manufacture's guidelines. For cDNA synthesis 1 μ g RNA was reverse-transcribed using QuantiTect reverse transcription kit (Qiagen). Reverse transcriptase quantitative real time polymerase chain reaction (RT-qPCR) was performed on an Applied Biosystems 7500 Fast Real Time System using SYBR green (Bioline: QT625-05). DNA oligo pairs for each gene are listed in Table S5. MMP-1 mRNA levels were normalized to GAPDH and determined relative to untreated cells using the 2^{- Δ ACt} method.^[22]

Determination of IC₅₀ Values Using MMP-1 Expression

MMP-1 mRNA expression level was determined as described above, normalized (starting value = 100 %) and plotted against log c of the tested compound (**B1**). To determine IC_{50} values, measured data was fitted against equation (2) using least squares regression (log (inhibitor) vs. response).

Crystallography

Protein purification and crystallization

Cloning, expression and purification of 14-3-3 σ was performed as described by Schumacher and colleagues.^[20] Compounds were available as 20 mM DMSO stock solutions. 14-3-3 σ at a concentration of 12 mg/ml was incubated overnight in the presence of 2 mM compound at 4°C. Crystallization was performed using the hanging-drop method at 4°C with 500 μ l crystallization buffer in the reservoir (crystallization buffers are listed in Table S3). Hanging-drops were mixed from 2 μ l complex solution with 2 μ l crystallization buffer from the reservoir. Due to the mother liquors cryogenic property the grown crystals were directly flash-cooled in liquid nitrogen.

X-ray Data Collection and Data Processing

Data collection was performed on in-house beamlines and at the Swiss Light Source (SLS) of the Paul Scherrer Institute, Villigen, Switzerland, beamline PXII. In-house beamlines were a Rigaku Micro-MAX-007 HF equipped with a MAR345dtb image plate and a Bruker AXS MICROSTAR equipped with a MAR345 image plate. Data processing was carried out with XDS.^[23] Crystal parameters and data collection statistics are listed in Table S4.

Crystal Structure Determination

CCP4 was used for model determination.^[24] Molecular replacement was carried out with PHASER using an unpublished structure of 14-3-3 σ as search model.^[25] The single solution was refined in iterative cycles of manual and automatic refinement with REFMAC and COOT.^[26,27] Refinement statistics are listed in Table S4. Simulated annealing composite-omit maps were calculated with PHENIX.^[28]



Figure S1. Docking poses for two different compounds $(14-3-3\sigma; \text{ grey SES}; \text{phosphate group from a peptide bound crystal structure: ball-and-stick model; docked compounds: stick models). a) Docking pose with phosphonate placement, which matches the spatially highly conserved position of the phosphate group of a sample peptide. b) Docking pose with highly deviating phosphonate placement as compared to the phosphate group of a sample peptide.$

Albeit docking poses and crystal structures for validated inhibitors considerably deviate (Figure S2), our strategy to prioritize compounds whose phosphonate poses matched the highly conserved phospho-recognition site was an effective selection criterion.



Figure S2. Residue conservation of all mammalian 14-3-3 isoforms mapped onto solvent-excluded surface of a $14-3-3\sigma$ structure. Color gradient from light-gray (highly conserved) to red (lowly conserved). The conservation of solvent exposed residues within the amphipathic groove surrounding superposed compound **B1** (stick-model) is 100%.



Figure S3. Complex structures. 14-3-3 σ (grey cartoon, phospho-coordinating residues as stick model) and compounds **B3-B11** (ball-and-stick models). Blue meshes are $2mF_o$ - DF_c simulated annealing composite-omit maps calculated with PHENIX.



Figure S4. Docking pose of **A1** (ball-and-stick model, yellow carbons) and its crystallized conformation (ball-and-stick model, grey carbons). a) 14-3-3 σ from docking structure (grey SES, PDB ID: 3P1N). Residues K49 and R60 are highlighted (green semi-transparent SES and stick models). Here, R60 is in a bent conformation and its guanidinium group partially occupies the space where the trifluoromethyl-benzene moiety of **A1** is located in the crystal structure. b) 14-3-3 σ (grey SES) from complex structure with **A1** (PDB ID: 3TOL). Residues K49 and R60 are highlighted (green semi-transparent SES and stick models). The conformation of R60 is elongated and flipped in comparison to the docking structure. Additionally, the highly flexible K49 adopts another main conformation.



Figure S5. 14-3-3 σ treatment stimulated MMP-1 in human lung fibroblasts (IMR-90). mRNA levels of MMP-1 were monitored by RT-qPCR, normalized to GAPDH and compared to untreated (PBS) cells. 14-3-3 σ increased MMP-1 expression in a concentration dependent manner. The highest tested concentration of 15 µg/ml revealed a nearly 3-fold induction and was used for further experiments.

ID	2D Structure	ID	2D Structure
A1		A8	
A2	HO'OH	A9	
A3	N O PO ₃ H ₂ OH N COOH	A10	о о но о но о но о но о но о но о но о но о но о но о но о но о но о но о но о но о но н
Α4		A11	
A5		A12	
A6		A13	HN O HO OH
Α7	HO OH	A14	

Table S1. Compound selection from virtual screening. A1 and A2 were validated as true actives.

Table S2. Active phenylphosphonic acid derivatives **B1-B11**. The inhibition curve from the FP assay is shown in red with the inhibition curve of compound **B1** in black as reference. Compounds **B12-B33** were found to have IC₅₀ values > 500 μ M and no FP curve is shown.







ID	R ¹	ID	R ¹
B12	CI	B25	CI CI
B13	F	B26	MeO
B14	OMe	B27	0 ^v vv _v
B15	- non	B28	COOMe
B16	OMe	B29	Inn
B17	CI	В30	O June
B18		B31	hun
B19	CI	B32	CI CI
B20	roor	B33	OMe
B21	OMe		
B22	- von		
B23			
B24	nor nor		

Table S3. Crystallization buffers.

Compound	B1	B2	B3	B4	B5 (A1)	B6	B7	B8 (A2)	B9	B10	B11
0.095 M HEPES Na	pH 7.1	pH 7.3	pH 7.5	pH 7.1	pH 7.7	pH 7.1	pH 7.5	pH 7.5	pH 7.1	pH 7.3	pH 7.7
PEG 400	27 %	28 %	28 %	28 %	28 %	27 %	26 %	26.6 %	28 %	28 %	28 %
CaCl ₂						0.19 M					
Glycerol						5 %					

Table S4. Data collection and refinement statistics.

Compound		B1	B2	B3	B4	B5 (A1)	B6		
PDB ID		4DHU	4DHT	4DHS	4DHR	3T0L	4DHQ		
Crystal parameters									
Cell dimensions	a, b, c (Å)	82.32, 112.46, 62,50	82.32, 112.34, 62.53	81.96, 111.95, 62.31	82.20, 112.34, 62.51	82.44, 112.79, 62.65	82.35, 112.39, 62.60		
	α, β, γ(°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0		
Space group		C222 ₁							
Data Collection ^a									
Beamline		Rigaku	SLS	Rigaku	SLS	SLS	Rigaku		
Wavelength (Å)		1.5418	0.9778	1.5418	1.0	0.9778	1.5418		
Resolution (Å)		19.54-1.67 (1.75-1.67)	45.52-1.80 (1.95-1.80)	19.47-1.74 (1.80-1.74)	45.49-1.40 (1.50-1.40)	45.62-1.6 (1.70-1.60)	19.56-1.75 (1.85-1.75)		
Measured reflecti	ons	262477 (21104)	103583 (20590)	257320 (15990)	275724 (39272)	338818 (54028)	206270 (20537)		
Unique reflection	s	33635 (4129)	26906 (5697)	28634 (2647)	56179 (10309)	38584 (6363)	29498 (4413)		
Completeness		98.8 (94.1)	98.7 (99.1)	96.1 (93.1)	98.2 (97.7)	99.2 (99.4)	99.3 (98.4)		
Redundancy		7,8 (5.1)	3.8 (3.6)	8.9 (6.0)	6.9 (5.4)	8.8 (8.5)	10.0 (4.7)		
ι / σ(Ι)		45.03 (13.16)	13.35 (4.14)	30.87 (6.78)	22.96 (4.06)	23.88 (5.85)	39.05 (13.71)		
<i>R_{meas}</i> (%) ^b		3.4 (13.3)	8.4 (36.0)	6.7 (31.9)	3.9 (34.8)	5.4 (43.0)	3.8 (11.5)		
Refinement ^{a,}									
Resolution (Å)		19.55-1.67 (1.71-1.67)	45.62-1.80 (1.85-1.80)	19.47-1.74 (1.78-1.74)	45.49-1.40 (1.44-1.40)	45.62-1.60 (1.64-1.60)	19.56-1.75 (1.80-1.75)		
Number of atoms		2525	2390	2417	2421	2460	2543		
R _{work} (%)		13.32 (16.80)	16.43 (22.40)	15.34 (21.70)	12.70 (18.10)	15.68 (19.20)	15.09 (19.30)		
R _{free} (%)		17.62 (24.60)	20.60 (29.80)	19.29 (26.80)	15.78 (24.0)	18.95 (22.70)	18.90 (23.70)		
RMS bond length	s (Å) ^c	0.019	0.024	0.018	0.026	0.021	0.017		
RMS bond angles	(°) ^c	1.718	1.924	1.646	2.301	1.997	1.571		
Averaged B-factors (Å ²)									
Total		16.91	20.19	16.41	19.46	22.32	16.29		
Compound		17.84	19,82	20.18	21.34	25.75	15.45		
Ramachandran plot residues									
Favored region	s (%)	97.1	96.2	97.1	97.6	96.2	96.1		
Add. allowed re	egions (%)	2.9	3.8	2.9	2.4	3.8	3.9		
Gen. allowed re	egions (%)	0.0	0.0	0.0	0.0	0.0	0.0		
Disallowed reg	ions (%)	0.0	0.0	0.0	0.0	0.0	0.0		

^a Data for the outermost resolution shells in parentheses.
 ^b Redundancy independent R-factor (intensities).^[29]
 ^c Root-mean square deviations (RMSD) from ideal geometry values.

Compound		B7	B8 (A2)	B9	B10	B11		
PDB ID		4DHP	3T0M	4DHO	4DHN	4DHM		
Crystal parameters								
Call dimensions	<i>a, b, c</i> (Å)	82.25, 112,23, 62,42	82.09, 112.30, 62.37	81.94, 111,69, 62.29	82.18, 111.80, 62.40	82.20, 112.28, 62.44		
Cell dimensions	<i>α, β,</i> γ(°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0		
Space group		C222 ₁						
Data Collection ^a								
Beamline		Rigaku	SLS, Rigaku	Rigaku	Rigaku	Bruker		
Wave length (Å)		1.5418	0.9778, 1.5418	1.5418	1.5418	1.5418		
Resolution (Å)		19.53-1.75 (1.85-1.75)	45.42-1.62 (1.70-1.62)	19.46-1.70 (1.85-1.70)	45.41-1.80 (1.80-1.90)	19.52-1.70 (1.85-1.70)		
Measured reflection	ons	234914 (22063)	245634 (21990)	166297 (23894)	134486 (14026)	176142 (23320)		
Unique reflections	;	28885 (4268)	36467 (4759)	31209 (6750)	26775 (3757)	31580 (6598)		
Completeness		97.9 (95.9)	98.6 (96.7)	98.0 (96.2)	99.0 (94.4)	98.2 (93.1)		
Redundancy		10.6 (6.8)	6.7 (4.6)	5.3 (3.5)	5.0 (3.7)	5.6 (3.5)		
ι / σ(I)		28.26 (7.27)	13.87 (4.04)	29.02 (8.01)	32.40 (12.03)	30.69 (9.73)		
<i>R_{meas}</i> (%) ^b		7.0 (27.7)	9.7 (43.7)	4.2 (17.0)	3.8 (11.7)	3.9 (15.1)		
Refinement ^a								
Resolution (Å)		19.53-1.75 (1.80-1.75)	45.42-1.62 (1.66-1.62)	19.46-1.70 (1.74-1.70)	45.41-1.80 (1.85-1.80)	19.52-1.70 (1.74-1.70)		
Number of atoms		2475	2467	2403	2412	2474		
R _{work} (%)		15.58 (20.20)	15.52 (19.50)	16.18 (19.50)	15.01 (18.40)	15.36 (21.80)		
R _{free} (%)		19.59 (25.20)	19.78 (24.80)	20.24 (23.10)	18.53 (24.00)	18.72 (23.10)		
RMS bond lengths	(Å) ^c	0.015	0.026	0.016	0.024	0.022		
RMS bond angles ((°) ^c	1.488	2.223	1.571	1.904	1.882		
Averaged B-factor	s (Ų)							
Total		15.66	20.17	16.42	18.12	17.01		
Compound		21.24	30.62	25.62	20.74	22.45		
Ramachandran plo	ot residues							
Favored regions	; (%)	95.6	95.2	96.6	97.1	96.2		
Add. allowed re	gions (%)	4.4	4.8	3.4	2.9	3.8		
Gen. allowed re	gions (%)	0.0	0.0	0.0	0.0	0.0		
Disallowed region	ons (%)	0.0	0.0	0.0	0.0	0.0		

^a Data for the outermost resolution shells in parentheses.
 ^b Redundancy independent R-factor (intensities).^[29]
 ^c Root-mean square deviations (RMSD) from ideal geometry values.

Table S5. Oligo pairs used in RT-qPCR.

	Sequence	Primer Bank Accession No.	Reference
GAPDH	5'-TGCACCACCAACTGCTTAGC-3'		[30]
	5'-GGCATGGACTGTGGTCATGAG-3'		[30]
MMP-1	5'-GGGGCTTTGATGTACCCTAGC-3'	225543092c3	[31–33]
	5'-TGTCACACGCTTTTGGGGTTT-3'	225543092c3	[31–33]

Table S6. PAMPA parameters measured for compounds **B1** and **B2**. Solubility value classifications: low (< 20), medium (20-120), high (> 120). PAMPA value classifications: low (< 5), medium (5-25), high (> 25).

Compound ID	Solubility [µM]	PAMPA [%Flux]
B1	470,9	-10,6
B2	471,4	-8,7

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