

Supporting Information

Nanomolar inhibitors of the peptidyl prolyl *cis/trans* isomerase Pin1 from combinatorial peptide libraries

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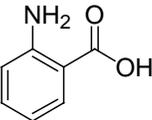
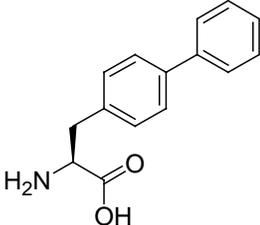
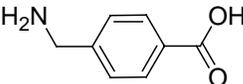
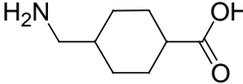
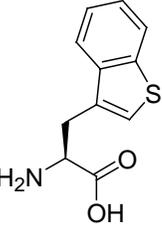
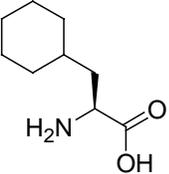
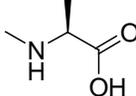
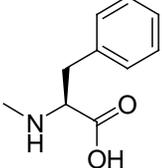
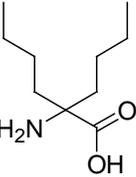
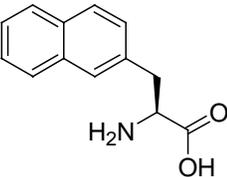
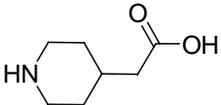
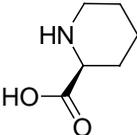
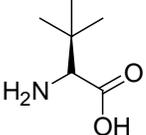
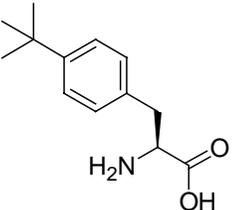
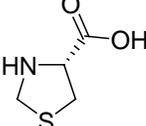
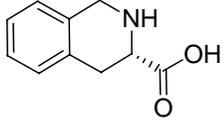
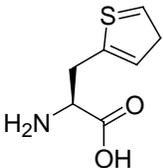
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Abbreviations:

ACN	Acetonitrile
calcd	calculated
DCM	Dichloromethane
EDT	Ethanedithiol
HATU	<i>O</i> -(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOAT	<i>N</i> -Hydroxy-9-azabenzotriazole
HOBT	<i>N</i> -Hydroxybenzotriazole
MS	Mass spectrometry
NMM	<i>N</i> -Methylmorpholine
PyBOP	Benzotriazole-1-yl-oxy-trispyrrolidino-phosphonium hexafluorophosphate
TFA	Trifluoroacetic acid
TIS	Triisopropylsilane
t _R	HPLC retention time

Supplementary Table 1. Chemical structures of the unnatural amino and *N*-alkylamino acids

H-Xaa-OH and H-Zaa-OH		H-Yaa-OH	
			
2-aminobenzoic acid H- Abz -OH	β -(4-biphenyl)-alanine H- Bip -OH	4-aminomethyl-benzoic acid H- Amb -OH	4-aminomethyl-cyclohexane- carboxylic acid H- Amc -OH
			
β -(3-benzothieryl)-alanine H- Bth -OH	β -cyclohexyl-alanine H- Cha -OH	<i>N</i> ^{α} -methyl-alanine H- MeAla -OH	<i>N</i> ^{α} -methyl-phenylalanine H- MePhe -OH
			
α, α -dibutyl-glycine H- Dbg -OH	β -(2-naphtyl)-alanine H- Nal -OH	4-piperidinyl-acetic acid H- Pia -OH	piperidine-2-carboxylic acid H- Pip -OH
			
α -t-butyl-glycine H- tBuGly -OH	4-t-butyl-phenylalanine H- tBuPhe -OH	thiazolidine-4-carboxylic acid H- Thz -OH	1,2,3,4-tetrahydro-isoquinoline- 3-carboxylic acid H- Tic -OH
			
β -(2-thienyl)-alanine H- Thi -OH			

Synthesis of the cellulose-bound combinatorial peptide library

The library was prepared by standard automated spot synthesis using an Auto-Spot Robot ASP 222 (Gilson) and Whatman 540 cellulose membranes.¹⁻³ The peptides were attached to the cellulose membrane via the side-chain of the C-terminal glutamine, which was achieved by coupling of Fmoc-Glu-OtBu to the amino group of the β -Ala- β -Ala-anchor group of the cellulose in the first cycle of the synthesis. Accordingly, a secondary amide bond was formed by condensation of the glutamic acid side chain and the free amino group of the β -Ala- β -Ala-anchor group resulting in a side chain *N*-alkylated glutamine residue at the C-terminus of the phosphopeptides.

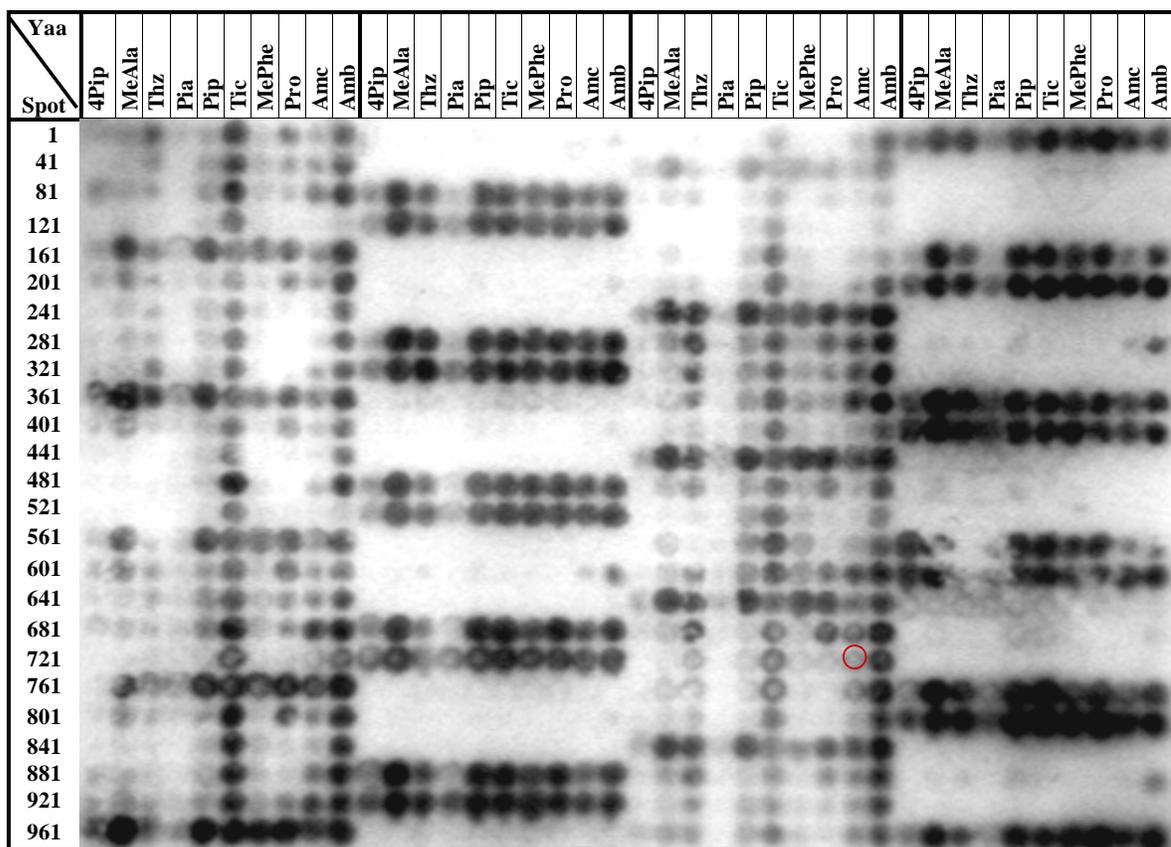
Screening of the cellulose-bound combinatorial peptide library for Pin1 ligands

Before screening, the cellulose-bound combinatorial peptide library was washed twice with methanol for 10 min followed by four times washing with binding buffer A (25 mM HEPES buffer, pH 7.5, 150 mM NaCl, 7.2 mM KCl, 2% glycerol, 1 mM DTT). The cellulose-bound combinatorial peptide library was then incubated with the *h*Pin1 PPIase domain (40 nM) in binding buffer A at 4 °C for one hour. After four washing steps at 4 °C in binding buffer A, the bound *h*Pin1 PPIase domain was electroblotted from the cellulose-bound combinatorial peptide library to a nitrocellulose membrane in a semidry blotting chamber (Biometra) at 96 mA. As transfer buffer 75 mM TRIS/HCl (pH 8.0) was used. The nitrocellulose membrane was then blocked by treatment with 20 mM TRIS/HCl buffer (pH 7.5) containing 150 mM NaCl, 0.05% Tween 20 and 3% BSA. Detection was done by incubation of the nitrocellulose membrane with an anti-*h*Pin1 PPIase domain antiserum from rabbit as primary antibody. As secondary antibody an anti-rabbit specific, horseradish peroxidase-conjugated antibody of goat serum was used (Dianova). Detection was done by a chemiluminescent reaction using the SuperSignal[®] substrate (Pierce).

The affinity of the *h*Pin1 PPIase domain regarding the phosphopeptides of the library was evaluated visually by comparison of spot intensity.

Screening results

Supplementary Figure 1A.



Supplementary Figure 1B.

Zaa \ Xaa	1-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100
1- 100	tBuPhe									
101- 200		tBuGly								
201- 300			Thi							
301- 400				Bth						
401- 500					Cha					
501- 600						Dbg				
601- 700							Bip			
701- 800								Abz		
801- 900									Phe	
901-1000										Nal

1A: The library was created by standard automated Spot synthesis on a cellulose membrane. The library composed of 1000 peptides with the general structure Ac-Xaa-Thr(PO₃H₂)-Yaa-Zaa-NHCH((CH₂)₂CONH-linker)COOH. The peptides were cellulose-anchored via a secondary amide bond formed by condensation of the amino group of the cellulose matrix bond β-alanyl-β-alanine linker and the carboxyl group of the glutamic acid side chain of the peptides. This procedure converts the C-terminal glutamic acid of the peptides to an N-alkylated glutamine residue. Screening was performed by incubation of the library with the recombinant *hPin1* PPIase domain followed by electroblotting of the bound protein to a nitrocellulose membrane. The *hPin1* PPIase domain bound to the spots was then detected using an anti-*hPin1* PPIase domain antiserum (rabbit) as primary antibody and an anti-rabbit specific, horseradish peroxidase-conjugated secondary antibody. Visualization was done by a chemiluminescent reaction using the SuperSignal[®] substrate (Pierce). The numbers indicated on the left side of the figure represent the number of the first spot of each horizontal line. The residue Yaa can be read from the label on the top of the corresponding row (e.g. red circle: spot 749, Yaa = Amc).

1B: The table shows the sequence key for the positions Xaa and Zaa. The amino acid at position Xaa changes every 100 spots. Spot 749 belongs to the range of 701 to 800 (see on left side of the table). Accordingly, Spot 749 represents a phosphopeptide with Abz at position Xaa. Zaa changes every ten spots. Spot 749 belongs to the range between 41 and 50 (see top of the table). Hence, the amino acid at position Zaa is Cha.

Synthesis of soluble peptides

Reagents used for chemical synthesis

Dibenzyl-diisopropyl-phosphoramidite, *N*-methylaniline-trifluoroacetate, *t*-butyl-hydroperoxide and Pd(PPh₃)₄ were purchased from Aldrich, Sigma and Fluka. Fmoc-protected amino acids with exception of Fmoc-D-Thr(PO(OBzl)OH)-OH are all commercially available (Bachem, Advanced Chemtech, NovaBiochem, Fluka, Senn Chemicals AG). Trifunctional amino acids were protected of their side chains as follows: Gln(Trt), Thr(OtBu), D-Thr(OtBu), D-Thr(Trt), Thr(PO(OBzl)OH) and D-Thr(PO(OBzl)OH). Peptide synthesis was performed using a Rink Amide MBHA resin (NovaBiochem) and PyBOP/HOBT (NovaBiochem) or HATU/HOAT (Perseptive Biosystems) as activation reagents. All other reagents used were of the highest purity commercially available.

Peptides were synthesized using a Syro II multiple peptide synthesizer (MultiSynTech, Germany) on a Rink Amide MBHA resin. Acylation was performed using Fmoc protected amino acids (four equivalents), PyBOP (four equivalents) and NMM (eight equivalents). Fmoc-protected Thr/D-Thr- derivatives were activated by HATU (double coupling). Peptides were cleaved from the resin by treatment with 94% TFA/2.5% H₂O/2.5% EDT/1% TIS (v/v/v/v). Peptides were precipitated with diethylether, purified by preparative RP-HPLC, lyophilized and stored at -8 °C. Analytical and preparative RP-HPLC was performed on Sykam or Pharmacia HPLC systems (Germany) with linear gradients of solvent B (0.1% TFA in ACN) in solvent A (0.1% TFA in H₂O). Product identity was verified by MALDI-ToF or ESI mass spectrometry.

Peptides 16 and 20

Peptides **16** and **20** were synthesized using the building block Fmoc-D-Thr(PO(OBzl)OH)-OH, that was synthesized as described for the L-Thr- derivative ⁴ with some modifications: Fmoc-D-Thr-OAll was synthesized starting from Fmoc-D-Thr-OH (29.3 mmol) by reaction with allyl bromide (29.3 mmol) and diisopropyl ethylamine (29.3 mmol) in DMF at rt. The solvent was evaporated and the product was redissolved in ethyl acetate followed by basic and acidic extractions and crystallization from ethyl acetate/diisopropylether.

Yield: 10.3 g, 92%; MS: $[M+H^+]_{\text{calcd}} = 382.16$, $[M+H^+]_{\text{found}} = 382.0$; analytical HPLC: $t_R = 18.46$ min, HPLC conditions: gradient from 5 to 100% of solvent B (0.1% TFA in ACN) in solvent A (0.1% TFA in H₂O) in 30 min, flow rate: 1 ml/min, HPLC column: LiChroCART[®] (Merck) 125-4 RP 8

Fmoc-D-Thr-OAll (12.34 mmol), dibenzyl-diisopropyl-phosphoramidite (37 mmol) and *N*-methylaniline-trifluoroacetate (61.7 mmol) in 100 ml DMF were stirred for one hour at rt under an argon atmosphere. Oxidation was performed by addition of *t*-butyl-hydroperoxid in *n*-octane (40 mmol). Fmoc-D-Thr(PO(OBzl)₂)-OAll was purified by silica gel chromatography (DCM/MeOH, 19:1).

Yield: 6.54 g, 83%; MS: $[M+H^+]_{\text{calcd}} = 642.3$, $[M+H^+]_{\text{found}} = 642.2$; analytical HPLC: $t_R = 23.2$ min (HPLC conditions as described above)

The allyl ester deprotection of Fmoc-D-Thr(PO(OBzl)₂)-OAll (10 mmol) was achieved with Pd(PPh₃)₄ (0.40 mmol) and acetic acid (25 mmol) in 50 ml DCM. After evaporation of the solvent, the product was washed four times with brine and purified by silica gel chromatography (DCM/MeOH, 19:1)

Yield: 5.1 g, 85%; MS: $[M+H^+]_{\text{calcd}} = 602.2$, $[M+H^+]_{\text{found}} = 602.0$; analytical HPLC: $t_R = 20.9$ min (HPLC conditions as described above)

Selective deprotection of one benzyl ester group of Fmoc-D-Thr(PO(OBzl)₂)-OH (8.5 mmol) was achieved in 50 ml ACN by addition of two equivalents of sodium iodide. The resulting mono sodium salt of Fmoc-D-Thr(PO(OBzl)OH)-OH was precipitated from ethyl acetate/diisopropylether and purified by silica gel chromatography (DCM/MeOH, 10:1).

Yield: 3.7 g, 85%; $[M+H^+]_{\text{calcd}} = 512.2$, $[M+H^+]_{\text{found}} = 512.0$; analytical HPLC: $t_R = 13.15$ min (HPLC conditions as described above)

Peptide **17** was synthesized using the standard method for "global phosphorylation" of resin bound peptides and Fmoc-D-Thr(Trt)-OH as building block.⁵⁻⁹

Peptide purity and identity

Peptide	HPLC solvent gradient	t_R (min)	Purity %	Mass spectrometry	
	H ₂ O/ACN (0.05% TFA)			$[M+H]^+$ _{calcd}	$[M+H]^+$ _{found}
1	10-60% ACN	18.7	98	1362.6	1362.5
2	10-60% ACN	16.1	100	1338.5	1338.7
3	10-60% ACN	15.5	100	1318.6	1318.7
4	10-60% ACN	17.52	100	1362.54	1362.6
5	10-60% ACN	18.1	98	1388.6	1388.8
6	10-60% ACN	18.1	97	1382.6	1382.6
7	10-60% ACN	13.04	98	1272.6	1272.6
8	10-60% ACN	16.5	100	1312.6	1312.4
9	10-60% ACN	18.7	100	1350.6	1351.1
10	20-50% ACN	19.9	97	1300.5	1300.6
11	10-60% ACN	16.5	100	1300.6	1300.7
12	10-60% ACN	15.0	99	1300.5	1300.7
13	10-60% ACN	21.0	99	1430.6	1430.4
14	10-60% ACN	19.3	100	1374.6	1374.5
15	5-100% ACN	12.48	99	1376.6	1376.9
16	10-60% ACN	14.72	100	1376.6	1376.9
17	10-60% ACN	16.5	100	824,3	824.4
18	10-60% ACN	16.5	99	824.3	824.4
19	10-60 % ACN	17.8	99	880.3	880.4
20	15-65 % ACN	13.6	100	1320.6	1320.5

HPLC: Pharmacia LKB with the peak integration software "PC Integretion Pack" (Kontron Instruments); **conditions:** flow rate: 1 ml/min, duration of the HPLC analysis: 30 min, HPLC column: LiChroCART® (Merck) 125-4 RP 8. Peptide purities were approved using another HPLC column (EC 250/4 NUCLEOSIL 100-7 C6H5, Macherey-Nagel).

¹H-NMR spectroscopic characterization of peptide 17

The NMR sample contained 1.67 mM of **17** set to pH 7.83 in a H₂O/D₂O mixture (95:5 v/v). All NMR experiments were collected at 25 °C using a DRX500 spectrometer (Bruker, Rheinstetten, Germany). ¹H-NMR data of **17** were acquired with a 5 mm inverse triple-resonance probe that has XYZ-gradient capability at 500.13 MHz resonance frequency. Standard 1D and 2D pulse sequences were employed. All spectra were processed with the XWINNMR 3.5 software (Bruker) and referenced to external 2,2-dimethyl-2-silapentane-5-sulfonate.

peptide 17: Ac-Phe-D-Thr(PO₃H₂)-Pip-Nal-Gln-NH₂

Ac	1.97
Phe	7.83 (H ^N), 4.76 (H ^α), 3.27/3.06 (H ^β), 7.32 (H ^{2,6}), 7.42 (H ^{3,5}), 7.32 (H ⁴)
D-Thr(PO ₃ H ₂)	9.82 (H ^N), 4.51 (H ^α), 4.29 (H ^β), 1.17 (H ^γ)
Pip	5.07 (H ^α), 2.06/1.37 (H ^β), 1.27/0.38 (H ^γ), 1.14/0.84 (H ^δ), 3.79/2.18 (H ^ε)
Nal	8.13 (H ^N), 4.97 (H ^α), 3.39/3.11 (H ^β), 7.61 (H ¹), 7.39 (H ³), 7.91 (H ⁴), 7.92 (H ⁵), 7.54 (H ⁶), 7.57 (H ⁷), 7.87 (H ⁸)
Gln	8.20 (H ^N), 4.17 (H ^α), 2.05/1.87 (H ^β), 2.21 (H ^γ), 7.49/6.84 (H ^ε)
NH ₂	7.46/6.90

Determination of the *hPin1* and *hPin1* ΔWW protein concentration

Protein concentrations were determined using the extinction coefficient $\epsilon_{280} = 21030 \text{ M}^{-1} \text{ cm}^{-1}$ for *hPin1* and $\epsilon_{280} = 6979 \text{ M}^{-1} \text{ cm}^{-1}$ for the *hPin1* PPIase domain. Extinction coefficients were calculated using the program ProtParam Tool at ExPASy (SIB, Switzerland). All measurements were done at 10 °C in 35 mM HEPES buffer (pH 7.8).

Protease free PPIase assay

The determination of the kinetic constants of the *cis/trans* isomerization within the protease-free system was performed using fluorescence based measurements on a Hitachi F-3010 fluorescence spectrophotometer with Abz-Ala-Glu-Pro-Phe-NH-Np as substrate in 35 mM HEPES buffer (pH 7.8). The substrate stock solution was 12 mM in anhydrous trifluoroethanol/0.55 M LiCl. Measurements were initiated by addition of the substrate (final concentration 10 μM) to a pre-incubated (15 min) mixture of 8.8 nM *hPin1* and the corresponding inhibitory peptide of the respective concentration. The excitation wavelength was 320 nm (spectral bandwidth 3 nm) and emission was detected at 420 nm (spectral bandwidth 10 nm).

Data analysis was performed by single-exponential nonlinear regression using the SigmaPlot Scientific Graphing System Version 5.00 (Jandel Corp.). The apparent first order rate constant

k_{obs} was computed by nonlinear regression of the progress curve obtained for the respective measurement. The rate constant k_0 was determined in absence of Pin1. The first order rate constant k_{enz} was calculated from the equation $k_{enz} = k_{obs} - k_0$. IC_{50} and K_i values were determined by fitting the first order rate constants k_{enz} against the concentration of the inhibitory peptide by nonlinear regression according to Schutkowski et al. and a model for tight binding inhibitors, respectively.^{10, 11}

Protease coupled PPIase assay: Analysis of the influence of **16 and **17** on the PPIase activity of *hPar14*, *hCyp18* and FKBP12**

Measurements were performed at 10 °C in 35 mM HEPES buffer (pH 7.8) using a Hewlett-Packard 8452A Diode-Array spectrophotometer.

Kinetic measurements with *hPar14* were performed using the substrate Suc-Ala-Arg-Pro-Phe-NH-Np (final substrate concentration: 75 μ M). PPIase activities of *hCyp18* and FKBP12 were measured with Suc-Ala-Arg-Pro-Phe-NH-Np as substrate (final substrate concentration: 16 μ M). In every case, α -chymotrypsin was used as isomerspecific protease (final concentration in the sample: 60 μ M). The final concentration of the respective PPIase in the sample was 1.7 μ M for *hPar14*, 7 nM for FKBP12, and 9.7 nM for *hCyp18*. Peptide **16** and **17**, were each incubated for 15 min with the respective PPIase before the measurements were started by addition of the substrate and protease. Data were collected at 390 nm. No significantly degradation of **16** and **17** by the protease occurred within the data collection period.

Protein kinase and protein phosphatase activities

Protein kinase activities were measured using the myelin basic protein as substrate for ERK2, the RII peptide as substrate for PKA and the neurogranin peptide as substrate for PKC.

The activities of the protein phosphatases were measured using ³³P-Phosphorylase a as substrate for PP1 and PP2A and the ³³P-RII phosphopeptide as substrate for PP2B. The protein kinase or protein phosphatase was incubated at 30 °C for one hour with the respective substrate in presence or absence of 500 μ M **16** or **17**. The enzyme concentrations were adjusted to an activity level of 30% phosphorylation or dephosphorylation of the substrates in the inhibitor-free samples. The substrate-associated radioactivity was measured using a Microbeta Topcounter (Perkin Elmer, Germany) and calculated against the corresponding control.

Far Western blot related analysis of the binding of peptide **15 towards different *hPin1* variants and its single domains**

GST-*hPin1* variants, GST-*hPin1* PPIase and GST-*hPin1* WW domain were prepared as described previously.^{12, 13} GST-*hPin1* and its single domains were spotted at increasing amounts onto nitrocellulose membranes. Remaining binding sites were blocked by treatment with binding buffer A containing 5% milk powder. The nitrocellulose membranes were then incubated with a 1 μ M solution of **15** or 50 μ M solution of its nonphosphorylated derivative in binding buffer A. After three washing steps, the nitrocellulose membranes were incubated with 2 μ g/ml of horseradish peroxidase conjugated-streptavidin (Pierce) for 90 min. Specific bound **15** was detected by a chemiluminescent reaction using the SuperSignal[®] substrate (Pierce).

For the analysis of the binding of **15** to different GST-*hPin1* variants, the indicated amounts of **15** were spotted onto nitrocellulose membranes. Blocking and washing steps were as

described above. The nitrocellulose membranes were incubated with the respective GST-*hPin1* variant (40 nM in binding buffer A). To detect the bound protein specifically, the membranes were incubated with an anti-*hPin1* PPIase domain antiserum as primary antibody and horseradish peroxidase-conjugated secondary antibody. Visualization was done using the SuperSignal[®] substrate.

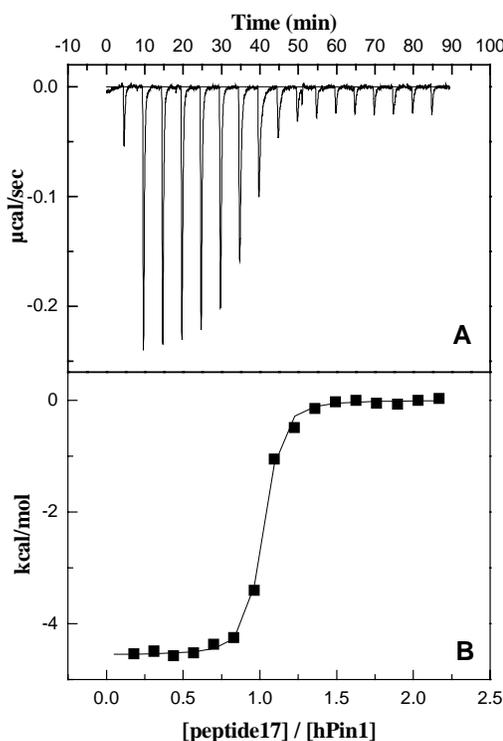
Capillary electrophoresis

Analysis was done using a P/ACE MDQ (Beckman Coulter, Palo Alto, CA) system. Fused-silica capillaries (Polymicro Technologies) of 40 cm length at an operation voltage of 20 kV were used. The samples were hydrodynamically injected with 3.45 kPa for 10 s. 50 mM sodium phosphate buffer (pH 7.0) was used as separation buffer. The system temperature was kept at 16 °C.

Isothermal titration calorimetry

Calorimetric experiments were performed using a VP-ITC titration calorimeter (MicroCal, Inc, Northampton, MA) at 10 °C. All solutions were degassed under vacuum prior use. Solutions of *h*Pin1 and *h*Pin1 PPIase domain were dialyzed against 10 mM HEPES (pH 7.8). The protein concentration in the sample cell was adjusted to 6.5 μ M using the same batch of buffer. The volume of the protein solution in the sample cell was 1.4 ml. The injection syringe was filled with 300 μ l solution of **17** (250 μ M in the same batch of buffer as used for dialyses). Each titration experiment consisted of a single 1 μ l injection followed by 16 identical injections of 5 μ l solution of **17**. Titration of the isolated PPIase domain of Pin1 was done analogical.

Supplementary Figure 2.



Thermodynamic characterization of the binding of **17** to full length *h*Pin1 by isothermal titration calorimetry

A: Peaks represent the heat evolved after injection of **17** into the sample cell containing *h*Pin1. After the 11th injection only the heat of dilution is observed. The experiment was performed at 10 °C.

B: Integrals of the corresponding peaks of panel A are plotted against the molar ratio of **17** and *h*Pin1. The solid line represents a nonlinear least squares fit analysis of the binding heat assuming a single binding site model. $n = 0.96$ (binding stoichiometry) and $K_A = 5.18 \times 10^7 \pm 7 \times 10^6 \text{ M}^{-1}$ (association constant). The dissociation constant K_D was calculated from the equation $K_D = 1/K_A$ with $K_D = 19.3 \pm 2.6 \text{ nM}$.

Cell culture and preparation of HeLa cell lysates

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, GibcoBRL) supplemented with 10% fetal calf serum at 37 °C with 5% CO₂. Cell lysates were prepared by treatment of the harvested cells with lysis buffer (25 mM HEPES buffer, pH 7.4, 150 mM NaCl, 7.2 mM KCl, 0.5% NP-40, 2% glycerol, 1 mM DTT) at 4 °C for 30 min followed by sonication and centrifugation at 13000 rpm. Depending on the respective experiment, phosphatase inhibitors (10 mM NaF, 1 mM Na₃VO₄, 0.5 μM okadaic acid) were added. After centrifugation at 15000 rpm at 4 °C the supernatants were used in the respective experiments.

Cell cycle analysis

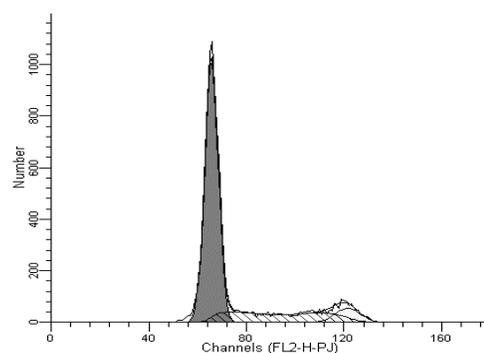
Cell cycle analysis was performed by determination of the intracellular DNA content with propidium iodide (PI). Cells were washed in PBS and fixed in ice-cold 70% ethanol for at least five hours. Before flow cytometry analysis cells were washed twice with cold PBS and rehydrated. The samples were treated with 30 μg/ml RNase A (Roche, Penzberg, Germany) at rt for at least two hours and stained with 50 μg/ml PI in the dark. The stained cells were analyzed by FACScan (Becton Dickinson) and fractions of cells in the different cell cycle stages were calculated using ModFit LT 3.1 software (Verity Software, Topsham, ME).

Transfer of peptide 17 into HeLa cells and determination of the cellular concentration

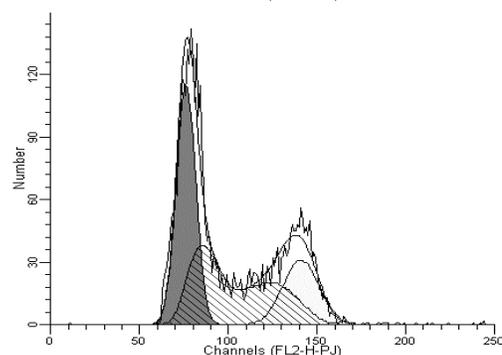
Peptide **17** was transferred into HeLa cells by electroporation using amaxa technology (Amaxa, Cologne, Germany). Different cellular concentrations of **17** (34 nM; 197 nM; 357 nM; 660 nM; 1,24 μM) were yielded using different concentrations of **17** during the transfection procedure (0.1 mM; 0.5 mM; 1 mM; 2 mM and 5 mM, respectively). Subsequently, the cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, GibcoBRL) supplemented with 10% fetal calf serum and 2 mM glutamine at 37 °C in a humidified incubator with 5% CO₂ atmosphere for three days. The cellular concentration of **17** was then determined by lysis of 5 x 10⁶ cells, respectively, in 100 μl 10 mM HEPES buffer (pH 7.4) followed by centrifugation and analysis of the corresponding supernatant by reversed phase HPLC. The cellular concentration of **17** was calculated by peak integration using a standard graph of **17**.

Supplementary Figure 3.

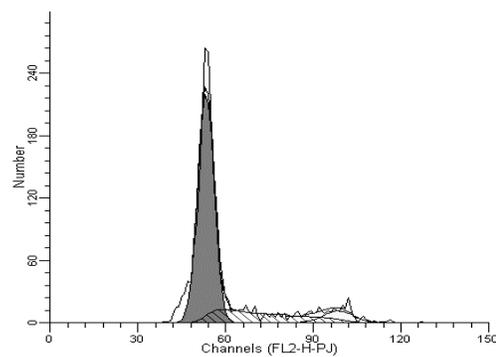
A



B



C



Specific inhibition of *hPin1* leads to an accumulation of HeLa cells in the G2/M phase
The FACS analysis has revealed a fraction of about 7% of the HeLa cells at the G2/M phase in absence of **17** (A). Figure B demonstrates the effect of *hPin1* inhibition by **17** on the cell cycle progression in HeLa cells. In presence of 1.24 μM of peptide **17** (cellular concentration) the fraction of cells at the G2/M phase was increased to 19.8%. In a control experiment (C) the nonphosphorylated derivative of **17** was transfected into the cells by electroporation resulting in a cellular concentration of 1.4 μM and as expected no influence on the cell cycle progression was observed.

dark gray: G0/G1 phase; with pattern: S phase; light gray: G2/M phase

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