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Designed Ankyrin Repeat Proteins (DARPins) as Novel Isoform-Specific Intracellular Inhibitors of c-Jun N-Terminal Kinases

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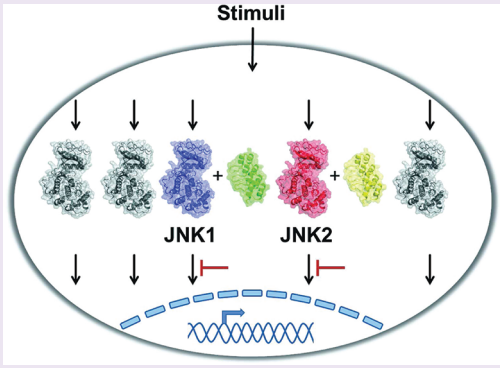
Supporting Information

ABSTRACT: The c-Jun N-terminal kinases (JNKs) are involved in many biological processes such as proliferation, differentiation, apoptosis, and inflammation and occur in highly similar isoforms in eukaryotic cells. Isoform-specific functions and diseases have been reported for individual JNK isoforms mainly from gene-knockout studies in mice. There is, however, a high demand for intracellular inhibitors with high selectivity to improve the understanding of isoform-specific mechanisms and for use as therapeutic tools. The commonly used JNK inhibitors are based on small molecules or peptides that often target the conserved ATP binding site or docking sites and thus show only moderate selectivity. To target novel binding epitopes, we used designed ankyrin repeat proteins (DARPins) to generate alternative intracellular JNK inhibitors that discriminate two very similar isoforms, JNK1 and JNK2. DARPins are small binding proteins that are well expressed, stable, and cysteine-free, which makes them ideal candidates for applications in the reducing intracellular environment. We performed ribosome display selections against JNK1α1 and JNK2α1 using highly diverse combinatorial libraries of DARPins. The selected binders specifically recognize either JNK1 or JNK2 or both isoforms *in vitro* and in mammalian cells. All analyzed DARPins show affinities in the low nanomolar range and isoform-specific inhibition of JNK activation *in vitro* at physiological ATP concentrations. Importantly, DARPins that selectively inhibit JNK activation in human cells were also identified. These results emphasize the great potential of DARPins as a novel class of highly specific intracellular inhibitors of distinct enzyme isoforms for use in biological studies and as possible therapeutic leads.



Mitogen-activated protein kinases (MAPKs) are key factors in cellular signal transduction and are involved in many important biological processes and a whole range of diseases. In mammalian cells, four major MAPK pathways have been described and named after their MAPK component: c-Jun N-terminal kinase (JNK),[1] extracellular signal-regulated protein kinase (ERK),[2] p38[3] and ERK5.[4]

The JNK pathway is preferentially activated by stressful and inflammatory stimuli including cytokines, growth factors, heat shock, oxidant stress and UV radiation and regulates a range of cellular processes that include proliferation, differentiation, apoptosis and inflammation.[5,6] JNKs are themselves activated by phosphorylation on two critical residues, Tyr and Thr, by the upstream MAPK kinases MKK4 and MKK7. Upon activation, JNKs regulate gene expression through phosphorylation of serine/threonine residues in discrete sets of transcription factors, such as c-Jun, ATF-2 and Elk-1. Furthermore, JNKs can activate various proteins in the cytoplasm and mitochondria and can thus regulate events in multiple intracellular compartments.[5,6]

In mammals, JNKs are encoded by three different genes (*jnk*1, *jnk*2 and *jnk*3) that are alternatively spliced to give rise to 10 highly similar isoforms. Both JNK1 and JNK2 are ubiquitously expressed, while JNK3 expression is restricted to the brain, heart and testis.[7] JNK1 and JNK2 occur as α and β variants, depending on differential splicing within protein kinase subdomains IX and X. Furthermore, they can also be alternatively spliced at the C-terminus resulting in short ("1" being appended to the name) or long ("2") splice forms, which can be detected as 46 kDa and 54 kDa protein products, respectively.[7] Sequence alignment of these different isoforms shows an amino acid identity of greater than 80%. *Jnk* gene knockout mice have provided valuable insights into the roles of individual JNK isoforms. Both JNK1 and JNK2 seem to be involved in the development of arthritis, heart disease and immune disorders (reviewed in ref 8). JNK1 has been shown to play a critical role in obesity, Type 2 diabetes and non-alcoholic liver disease, whereas JNK2 seems to be involved in atherosclerosis, Type 1 diabetes and TNF-induced liver damage.[8] To further elucidate isoform-specific physiological functions of JNK1 and JNK2 and to target isoform-specific

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diseases, selective inhibition of individual JNK isoforms is necessary.

The most commonly used JNK inhibitors are based on small molecules that target the active site of the enzyme, which is highly conserved among protein kinases and includes a binding site for the essential co-factor ATP (reviewed in refs 9 and 10). Specificity is thus difficult to achieve, and inhibition is further attenuated by the high ATP concentration in the cells that must be competed, which was also observed with SP600125, a widely used ATP-competitive inhibitor of JNKs.[11]

As an alternative, peptide inhibitors were generated that interfere with the docking sites of upper kinases or downstream substrates or the scaffold proteins on the surface of JNKs. These inhibitors have usually been derived directly from the sequences of these interacting partners of JNKs. For example, cell-permeable peptides derived from the scaffold protein JNK-interacting protein 1 (JIP1) were successfully used in studies for JNK-related diseases such as diabetes, stroke and neurodegenerative diseases (reviewed in ref 12). Recently, a novel JNK inhibitory peptide, PYC71N, was identified that inhibits the binding of JNK to c-Jun.[13]

The binding of small molecules occurs preferably in grooves and cavities and often shows only moderate affinities, probably due to the small interaction interface. Peptide-based inhibitors are mostly directed against known docking sites, and to our knowledge, selectivity between different JNK isoforms has not been achieved so far. Protein-based inhibitors are a promising alternative to small molecules and peptide-based inhibitors, because protein−protein interactions involve larger interaction interfaces that could result in inhibitors with higher specificity and higher affinity and, most likely, a different inhibition mode. "Intrabodies" have been mostly based on single-chain Fv antibodies (scFvs), but they often lack sufficient stability inside the cell, since many scFvs require disulfide bonds, which cannot form in the cytoplasm. This problem can be solved with rational and evolutionary engineering efforts or by restriction to a few well folding subtypes in scFv format, as single domains or as camelid VHH domains.[14−17] Alternatively, high specificity and intracellular activity can be achieved when starting from frameworks that do not require disulfides for stability, as exemplified, *e.g.*, with the fibronectin scaffold (monobodies) directed against a small ubiquitin-related modifier (SUMO) 1 isoform and against the Abl SH2 domain, respectively.[18,19]

We have developed the technology of designed ankyrin repeat proteins (DARPins), which provide a large rigid interaction surface, are very stable and do not have any cysteines.[20,21] They are predestined to bind to the surface of target proteins with high specificity and thus provide an attractive approach to target novel epitopes on the surface of JNKs and to contribute to the development of isoform-selective inhibitors that discriminate between JNK1 and JNK2.

DARPins are based on naturally occurring ankyrin repeat proteins that are an abundant class of binding proteins found in nearly all phyla.[22] Ankyrin repeats are present extracellularly, intracellularly and in membrane-associated form and are involved in many diverse biological processes such as cell cycle control, transcriptional regulation, cytoskeletal organization, cell development and differentiation.[22,23] Based on sequence and structure analysis, combinatorial libraries of DARPins were designed with very favorable biophysical properties. They are very well expressed and soluble in the bacterial cytoplasm, highly stable and cysteine-free, which makes them interesting candidates for applications in the reducing intracellular environment.[20,24] DARPin libraries, in combination with powerful selection techniques such as ribosome display[25] or phage display,[26] have already been used successfully to obtain target-specific binders with high affinities, *e.g.*, to MAPKs, members of the ErbB receptor family, TNFα and epithelial cell adhesion molecule (EpCAM).[27−30] Furthermore, DARPins were created that show intracellular inhibition of the bacterial kinase aminoglycoside phosphotransferase type (3′)-IIIa (APH) by binding to the C-terminal loop of the kinase and trapping it in an inactive conformation.[31,32] Other selected DARPins inhibit the TNF-α-mediated NF-κB activation in human cells.[33]

In this work, we describe the rapid selections of highly selective DARPins that recognize either exclusively JNK1 or JNK2 or both isoforms *in vitro* with affinities in the low nanomolar range. All analyzed DARPins inhibit the activation of JNK in an isoform-specific manner *in vitro* at physiological ATP concentrations. Furthermore, selected DARPins show isoform-specific binding to JNK1 and JNK2 and inhibition of JNK activation in mammalian cells.

RESULTS AND DISCUSSION

Earlier studies have demonstrated that DARPins can be selected to virtually any target of interest with high specificities and high affinities. Since they express and fold well in the cytoplasm, we wanted to test whether DARPins could be used as specific intracellular modulators of enzymes or other proteins in general. In the present work, we applied a competition ribosome display selection approach to identify DARPins that discriminate between the two highly similar isoforms JNK1 and JNK2. Binding specificities were determined *in vitro*, in cell lysates as well as within mammalian cells using bioluminescence resonance energy transfer (BRET) technology. Finally, inhibition of the JNK pathway was analyzed *in vitro* with an ELISA-based kinase assay and, as the most critical test, in human cells.

Selections and Binding Specificities of JNK Isoform-Specific DARPins. In eukaryotic cells, the most dominant isoforms found are the short splice form of JNK1 (JNK1α1) and the long splice form of JNK2 (JNK2α2).[34−37] For the generation of isoform-selective binders we chose the short α splice forms JNK1α1 and JNK2α1 as targets to direct the selections toward binders that can discriminate between JNK1 and JNK2 by binding to the kinase core instead of the elongated C-terminus of the long splice form. Non-biotinylated and biotinylated JNKs were produced in *E. coli* in a soluble and non-phosphorylated (inactive) form as fusions to bacteriophage lambda protein D (pD)[38] to increase expression yield and solubility of the targets, as described previously.[27] The target pD_JNK1α1 could be purified as a monomeric protein, whereas pD_JNK2α1 was present as a mixture of monomers and dimers, which could not be separated due to rapid equilibration between these two species.

For selections, the highly diverse combinatorial DARPin libraries N2C and N3C were used, which consist of an N-terminal capping repeat, 2 or 3 internal repeats with randomized positions and a C-terminal capping repeat.[20] These libraries were applied to ribosome display (RD) selections, a method that works entirely *in vitro* linking genotype and phenotype *via* the ribosome.[25] To increase selection pressure for isoform specificity, the target isoform pD_JNK1α1 was immobilized on plates and the non-desired isoform pD_JNK2α1 was present as competitor in solution and

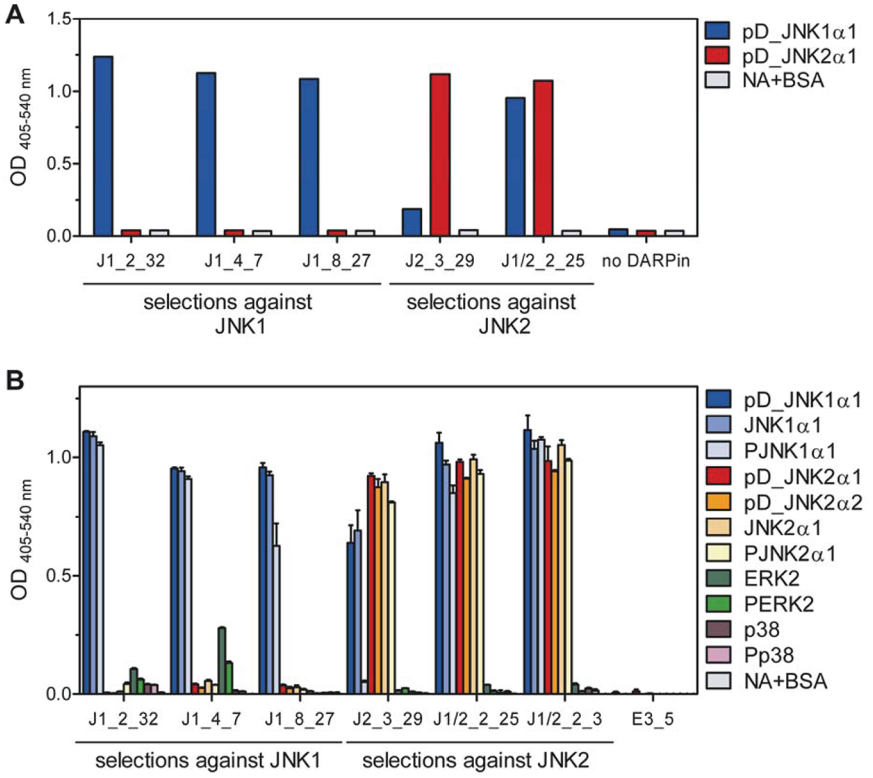

Figure 1. Binding specificities of selected DARPins. (A) DARPins derived from ribosome display selections were tested in a crude extract ELISA for specific binding to JNK1 and JNK2 and neutravidin + BSA as control. (B) Purified DARPins (200 nM) were analyzed for isoform-specific interactions as well as for binding to other MAPKs. To rule out unspecific binding to pD, MAPKs lacking pD fusion were tested. Binding of DARPins to active phosphorylated (P) MAPKs was also investigated. DARPin J1/2_2_3 that was previously selected against JNK2 (original name JNK2_2_3) and the unselected library member E3_5 were also included. The experiment was performed in triplicates, and the background binding signals from detection antibodies were subtracted.

vice versa. Already after 3 RD selection rounds, enrichment for the targeted JNK isoform with the N2C and N3C libraries was observed for both targets, compared to the unspecific background control neutravidin and bovine serum albumin (BSA) (Supplementary Figure S1).

To screen individual DARPins for binding to their specific target, single clones were expressed in *E. coli* and analyzed using an enzyme-linked immunosorbent assay (ELISA) with crude *E. coli* extracts as described previously.[29] From the selections against JNK1α1, 40% N2C DARPins and 47% N3C DARPins were identified that showed specific binding to JNK1α1. Only few DARPins were found that bound to both isoforms (4% N2C and 2% N3C DARPins). Selections against the other isoform JNK2α1 resulted in 56% N2C DARPins and 40% N3C DARPins with specific binding signals for the targeted isoform. In addition, 33% N2C DARPins and 26% N3C DARPins were identified that recognized both JNK1 and JNK2. None of the analyzed DARPins showed binding to the neutravidin + BSA control. DARPins with the most prominent binding signals in the crude extract ELISA are presented in Figure 1A. The DARPins were named J1_#, J2_# or J1/2_#, according to their binding specificities to JNK1, JNK2 and both isoforms, respectively.

To determine the binding specificities of the selected DARPins in more detail, interactions with different JNK isoforms and other highly similar MAPKs, such as ERK2 and p38α, were investigated by ELISA using purified DARPins (Figure 1B). To exclude unspecific binding to the fusion protein pD, interactions with MAPKs lacking pD were also analyzed. Furthermore, we included the phosphorylated (active) forms of the kinases to test whether the selected DARPins could also recognize the active state of a kinase.

All selected DARPins showed specific binding to JNK isoforms but no (or little, in case of J1_4_7) interaction with the other highly similar MAPK ERK2 or p38α, demonstrating a high binding specificity for JNKs. Furthermore, the recognition of JNK isoforms was independent of the presence of the fusion protein pD, confirming the exclusive binding to the target JNKs. JNK1-specific DARPins, namely, J1_2_32, J1_4_7 and to a lesser extent J1_8_27, also recognized the active phosphorylated form of their target, but none of them interacted with any analyzed JNK2 isoform. DARPin J1/2_2_25 targeted all tested JNK isoforms. Surprisingly, DARPin J2_3_29 showed binding not only to all JNK2 isoforms but also some significant binding to non-phosphorylated JNK1 under these assay conditions (Figure 1B), which is in contrast to the results obtained from the crude extract ELISA (Figure 1A). Nevertheless, DARPin J2_3_29 did not bind to phosphorylated JNK1. Therefore, the selectivity for JNK2 was at least maintained in the case of the active kinases. Unfortunately, other analyzed DARPins that showed JNK2-specific binding signals in the crude extract ELISA failed to discriminate between JNK1 and JNK2 under these conditions. The most likely explanation for this seems to be the presence of detergents in the crude extract ELISA that denatured the corresponding binding epitopes on JNK1, leading to the impression of JNK2 specificity.

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We also included DARPin J1/2_2_3 (original name JNK2_2_3[27]) in our studies. This DARPin was previously selected against JNK2 and showed specific binding to JNK2 with low nanomolar affinity but had not been tested for binding to JNK1.[27] In our more detailed analysis, DARPin J1/2_2_3 recognized all tested isoforms of JNK2 as well as of JNK1 (Figure 1B). Therefore, the name was changed to J1/2_2_3 to demonstrate that this DARPin can bind to both isoforms and not only to JNK2. Interestingly, DARPin J1/2_2_3 has the same binding specificity as J1/2_2_25, even though they were obtained from independent selections using different strategies. Furthermore, looking at the amino acid sequences, DARPins J1/2_2_25 and J1/2_2_3 show very high similarity, whereas the other DARPins have very distinct sequences (Supplementary Figure S2). The unselected DARPin library member E3_5 was included as negative control and showed no binding to any kinase, as expected.

In summary, the selections against two highly similar JNK isoforms JNK1α1 and JNK2α1 resulted in DARPins that either exclusively recognize JNK1 or demonstrate preference to JNK2 or bind to both isoforms. All DARPins are very selective for the JNK family members and do not bind to other MAP kinases.

Size Exclusion Chromatography of DARPins and Stoichiometry of JNK-DARPin Complexes. Since a tendency for aggregation would be an undesired property of a potential inhibitor, the selected DARPins were further characterized by size exclusion chromatography (SEC). All selected binders eluted as a single peak, suggesting no aggregation tendency in solution at a concentration of 15 μM (Supplementary Figure S3).

To determine the exact molar mass of the DARPins and the stoichiometry of JNK-DARPin complexes, SEC combined with multi-angle (static) light scattering (MALS) was performed. In case of JNK1, the kinase alone eluted as a single monomeric species (Figure 2A,B). Upon binding of the selected DARPins, the peak of the corresponding complex shifted to lower elution volume and the molar masses of the complexes were determined to be about 71.8 kDa in the presence of N2C DARPins and about 75.2 kDa in the presence of N3C DARPins (theoretical molar mass for pD_JNK1α1 is 57 kDa, for a N2C DARPin about 14.8 kDa, for a N3C DARPin about 18.2 kDa). This indicates a 1:1 binding stoichiometry for all JNK1-DARPin complexes (Figure 2A,B). Additional small peaks were observed at lower elution volume with calculated masses of about 130−168 kDa, which would imply a small amount of dimer formation of the whole complexes.

Similar findings were obtained for the JNK2-DARPin complexes (Figure 2C). All analyzed DARPins bound in a 1:1 ratio to the monomeric species of the kinase, resulting in a protein complex of 71.6 kDa (theoretical molar mass for pD_JNK2α1 is 56.8 kDa, for a N2C DARPin about 14.8 kDa). The JNK2 kinase alone showed the expected monomeric peak, but in contrast to JNK1 also an additional peak at 109.5 kDa, indicating a dimeric state. Upon binding of the selected DARPins, this dimeric peak showed also a slight shift to lower elution volume and calculated molar masses of 115−140 kDa, which suggests a binding of the DARPins to the dimeric kinase as well (Figure 2C). As for JNK1, additional small peaks at lower elution volume were observed after formation of JNK2-DARPin complexes, implying a slight tendency for complex oligomerization. An approximate molar mass could not be determined due to the low concentration of these oligomers. The molar masses calculated from the peaks corresponding to free DARPins confirmed the monomeric state for all analyzed DARPins (Figure 2A−C).

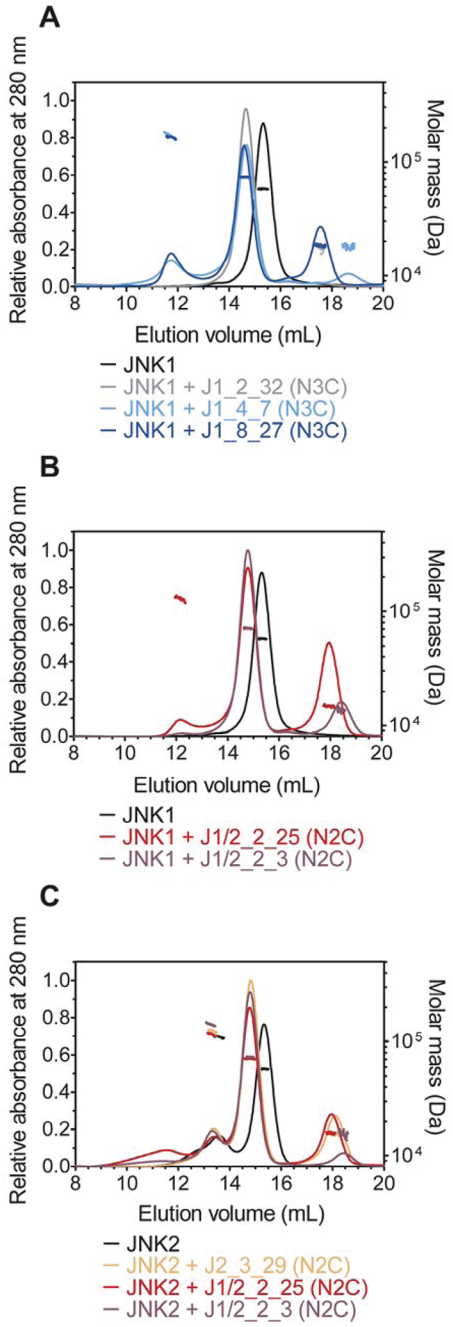

Figure 2. Determination of stoichiometry of JNK-DARPin complexes. Elution profiles and molar masses of JNK1 (A, B) and JNK2 (C) alone and in complex with DARPins were analyzed by size-exclusion chromatography combined with multi-angle (static) light scattering (SEC-MALS). pD_JNK1α1 or pD_JNK2α1 (15 μM) were incubated with the corresponding DARPin (37.5 μM) at a molar ratio of 1:2.5, and the mixtures were loaded on a Superdex 200 column. The elution profiles are monitored by the relative absorbance at 280 nm (left-hand *y*-axis). The determined molar masses are given by the horizontal traces at the corresponding protein peaks (right-hand *y*-axis). For each DARPin, the number of repeats is given in parentheses. Note that the number of aromatic amino acids is different in these DARPins (Supplementary Figure S2), which leads to different signal intensities at 280 nm.

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Table 1. Affinities and Binding Kinetics of Selected DARPins[a]

DARPin (size)	target	k_{on1} (10^5 M^{-1} s^{-1})	k_{off1} (10^{-4} s^{-1})	K_{D1} (10^{-9} M)	k_{on2} (10^5 M^{-1} s^{-1})	k_{off2} (10^{-4} s^{-1})	K_{D2} (10^{-9} M)
J1_2_32 (N3C)	JNK1α1	2.61 ± 0.04	48.7 ± 0.6	18.7 ± 0.4	9.05 ± 0.36	13.3 ± 0.5	1.47 ± 0.08
	JNK2α1	NB	NB	NB	NB	NB	NB
J1_4_7 (N3C)	JNK1α1	3.26 ± 0.03	9.11 ± 0.10	2.79 ± 0.04	21.3 ± 0.9	44.4 ± 1.2	2.08 ± 0.10
	JNK2α1	NB	NB	NB	NB	NB	NB
J1_8_27 (N3C)	JNK1α1	13.1 ± 0.2	84.4 ± 0.9	6.44 ± 0.12	2.23 ± 0.02	3.10 ± 0.09	1.39 ± 0.04
	JNK2α1	NB	NB	NB	NB	NB	NB
J2_3_29 (N2C)	JNK1α1	NB	NB	NB	NB	NB	NB
	JNK2α1	3.12 ± 0.07	42.8 ± 0.4	13.7 ± 0.3	19.4 ± 0.9	20.7 ± 0.5	1.07 ± 0.06
J1/2_2_25 (N2C)	JNK1α1	16.9 ± 0.3	46.5 ± 0.7	2.75 ± 0.06	4.16 ± 0.04	8.31 ± 0.13	2.00 ± 0.04
	JNK2α1	3.03 ± 0.04	7.39 ± 0.06	2.44 ± 0.04	26.9 ± 0.4	14.5 ± 0.1	0.539 ± 0.01
J1/2_2_3[b] (N2C)	JNK1α1	4.07 ± 0.06	12.0 ± 0.1	2.95 ± 0.05	65.5 ± 1.9	6.91 ± 0.12	0.105 ± 0.004
	JNK2α1	1.97 ± 0.03	9.30 ± 0.08	4.72 ± 0.07	32.4 ± 0.3	9.30 ± 0.06	0.287 ± 0.004

[a]SRP binding kinetics were measured at 15 °C and fitted with a heterogeneous ligand model (see Supporting Information). NB: no binding to the target detected. Errors represent statistical fitting errors. [b]Original DARPin name JNK2_2_3. Binding constants for JNK2α1: k_{on} = 9.7 × 10^5 M^{-1} s^{-1}, k_{off} = 20 × 10^{-4} s^{-1}, K_D = 2.1 × 10^{-9} M.[27]

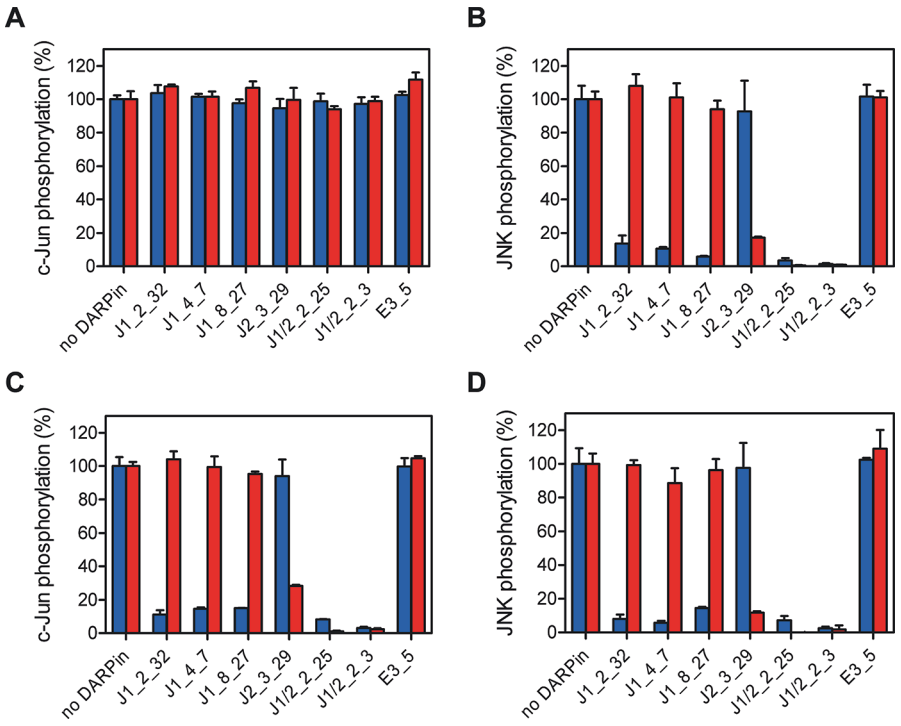

Figure 3. *In vitro* inhibition properties of selected DARPins. ELISA-based kinase assays were carried out, and the amount of phosphorylated JNK or phosphorylated substrate c-Jun was detected with phospho-specific antibodies against JNK or c-Jun. Graphs with blue bars belong to the JNK1 pathway, and graphs with red bars belong to the JNK2 pathway. (A) To investigate any direct influence of selected DARPins on JNK enzyme activity, each DARPin (250 nM) was mixed with either active JNK1α1 or active JNK2α1 (4 nM) and the phosphorylation of c-Jun was assayed. (B, C) To examine the inhibitory effect of selected DARPins on the activation of JNK1 and JNK2 themselves, inactive (non-phosphorylated) pD_JNK1α1 or inactive (non-phosphorylated) pD_JNK2α1 (4 nM) were incubated with the indicated DARPins (250 nM) and the phosphorylation of JNK1 and JNK2 was monitored after incubation with the active upper kinase MKK4 (B). The same samples were further tested for phosphorylation of c-Jun by JNK1 or JNK2 (C). (D) For inhibition of JNK activation in a cellular context, either inactive pD_JNK1α1 or inactive pD_JNK2α1 (4 nM) was mixed with the indicated DARPins (250 nM) and the phosphorylation of JNK1 and JNK2 was initiated with activated cell extract from HEK 293T cells. As control for all experiments, the unselected library member E3_5 (250 nM) was included. All experiments were performed in triplicates, and the background signal of the detection antibodies was not subtracted. The amount of phosphorylated JNK or c-Jun with no DARPin present was set to 100%.

Binding Constants of Selected DARPins. The affinities of the selected binders toward both targets JNK1α1 and JNK2α1 were analyzed by surface plasmon resonance (SPR), and the data were evaluated with a heterogeneous ligand model (Table 1 and Supplementary Figure S4). This model seemed to be most suitable and could be rationalized by the 1:1 binding stoichiometry of the complexes, the poor stability of JNK1, the heterogeneity of JNK2 and the flexibility of the kinases in general. All selected DARPins bound their specific target(s) with affinities in the low nanomolar range (Table 1). Interestingly, DARPin J2_3_29 bound selectively to JNK2α1 and not to JNK1α1 under these conditions, which might be due to lower concentrations or slightly different buffer composition

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compared to the conditions used in the ELISA experiment (Figure 1B).

***In Vitro* Inhibition Properties of Selected DARPins.** To investigate whether the selected DARPins inhibit kinase *activation* and/or kinase *activity in vitro*, we performed ELISA-based enzyme assays as described previously[39] with some modifications. The phosphorylation of JNK itself (JNK activation) was monitored by a JNK-phospho-specific antibody, while the phosphorylation of the JNK substrate c-Jun (JNK activity) was measured using a c-Jun-phospho-specific antibody. For all assays, 1 mM ATP was used to mimic the physiological ATP concentration in cells and to screen for potential ATP-noncompetitive allosteric inhibition mode.

To examine the direct influence of selected DARPins on JNK activity, each DARPin was mixed with either active JNK1α1 or active JNK2α1, and the phosphorylation of c-Jun was assayed (Figure 3A). As controls, JNK1 and JNK2 activities were measured in the absence of DARPins and in the presence of the unselected DARPin library member E3_5. Under these conditions, no significant inhibition of JNK activity was observed. This implies that the selected DARPins cannot directly prevent activated JNK from phosphorylating its substrate, indicating that they do not interfere with substrate binding or catalysis.

To investigate the inhibitory potential of selected DARPins on the activation of JNK1 and JNK2 themselves, non-phosphorylated JNK1 and JNK2, respectively, were first incubated with corresponding DARPins. Subsequently, the JNK activation reaction was started with active kinase MKK4, which is the upstream kinase of JNK1 and JNK2, and the rate of JNK phosphorylation was measured (Figure 3B). After JNK activation, part of the samples was further used to test for phosphorylation of c-Jun in a second kinase reaction (Figure 3C). At the level of JNK activation, DARPins J1_2_32, J1_4_7 and J1_8_27 specifically reduced JNK1 phosphorylation to 5–13%, whereas J2_3_29 only significantly inhibited the JNK2 isoform (17% remaining activity). The most potent inhibitors turned out to be J1/2_2_25 and J1/2_2_3, which showed almost complete inhibition toward both JNK isoforms (0.6–3.5% residual activity) (Figure 3B). The inhibition of JNK activation by the DARPins also prevented JNK from phosphorylating its substrate c-Jun (Figure 3C). No inhibition of JNK activation or JNK activity was observed with the control DARPin E3_5 (Figure 3B,C). Thus, the overall inhibition mode of all inhibiting DARPins seems to be at the level of JNK activation, which results in a downstream inhibition effect toward its substrate c-Jun.

To investigate this inhibitory effect in a cellular context, kinase assays with activated cell extract from human embryonal kidney 293 T (HEK 293T) cells were performed. For this purpose, JNK1α1 and JNK2α1 were again first mixed with the individual DARPins, and after activation with activated cell extract (instead of active purified MKK4), the rate of JNK phosphorylation was monitored. Under these conditions, the individual DARPins demonstrated the same isoform-specific inhibition of JNK activation as with purified MKK4, with remaining residual activities ranging from 0 to 14%, depending on the respective DARPin, whereas control DARPin E3_5 had no detectable effect (Figure 3D).

We thus demonstrated that all selected DARPins exhibit strong inhibitory effects with high selectivity toward either JNK1 or JNK2 or both JNK isoforms under physiological ATP concentration. Interestingly, all inhibitory DARPins showed the same inhibition mode, protecting the kinase from being phosphorylated itself, and this effect was moreover independent of their isoform-specificity. For the best inhibiting DARPins J1/2_2_25 and J1/2_2_3, almost complete inhibition was observed at the protein level, comparable with gene knockouts.

These findings suggest that the inhibitory DARPins act as inhibitors at the level of JNK activation by either preventing binding of the upper kinase(s) MKK4/7 to JNK or by interfering with the activation process itself, *e.g.*, by an allosteric effect. Compared to small molecule and peptide inhibitors that target the ATP binding site or the substrate binding site, the selected DARPins thus provide a different inhibition mode. This inhibition mechanism, combined with high selectivity and affinity, makes them interesting candidates for validation and modulation of the JNK pathway.

In general, these findings support the vision that DARPins combined with powerful selection strategies can be targeted against various regions on a kinase and thereby can provide distinct inhibition mechanisms. The selected DARPins presented in this study inhibit JNK activation and thus seem to have a distinct inhibition mode when compared to DARPins selected against other kinases. For example, DARPin AR_3a that inhibits the bacterial kinase aminoglycoside phosphotransferase type (3′)-IIIa (APH) binds to the C-terminal lobe of the kinase and induces a structural change in the substrate binding site. This results in allosteric inhibition of APH kinase activity.[31,32] We recently developed DARPins that can discriminate between non-phosphorylated (inactive) and phosphorylated (active) forms of another MAPK, namely, ERK2. They bind to the region of the phosphorylation loop and should thereby lead to different inhibition modes.[40] Thus, DARPins can contribute to the identification of various novel epitopes leading to potential new inhibition mechanisms.

Binding Specificities to Endogenous JNKs. To analyze the binding of selected DARPins to endogenous JNK isoforms directly from human cells, pull-down experiments were performed as described previously[41] with some adaptations. For this purpose, endogenous JNK isoforms were immunoprecipitated from HEK 293T cell lysate by individual DARPins coupled to Ni-NTA beads and then analyzed by Western blotting. For detection of specific JNK isoforms, an antibody against JNK1 and an antibody against JNK2 were used. The presence of the DARPins was confirmed with an antibody recognizing the N-terminal His-tag of the DARPins (Figure 4). All JNK1-specific DARPins showed a band in the JNK1-specific Western blot but not in the JNK2-specific Western blot, indicating that they can specifically bind to endogenous JNK1. Nevertheless, the intensities of the bands differed significantly between the individual DARPins, with J1_8_27 being the most potent DARPin for capturing endogenous JNK1 (Figure 4). Interestingly, even though the JNK1-specific antibody can recognize both the short (p46) and the long (p54) splice forms of JNK1, only the p46 splice forms were detectable in the pull-down samples as well as in the cell lysate itself, suggesting that the p46 splice forms are the predominant JNK1 splice forms in these cells. DARPin J2_3_29 precipitated only JNK2 and not JNK1, supporting its binding specificity for the JNK2 isoform. Since the JNK2-specific antibody only recognizes the long JNK2 splice forms, no conclusions can be drawn which splice forms of JNK2 are present in the cells. DARPins J1/2_2_25 and J1/2_2_3 precipitated both JNK isoforms from the cell lysate, giving rise to the corresponding bands on the JNK1- and JNK2-specific Western blots. Since with the control DARPin

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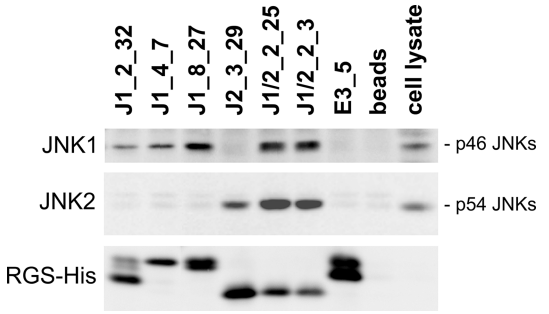

Figure 4. Binding of selected DARPins to endogenous JNKs. In a pull-down experiment, endogenous JNK isoforms were immunoprecipitated from HEK 293T cells by selected DARPins and analyzed by Western blotting. For detection, specific antibodies against JNK1 (both splice forms) and JNK2 (long slice form) were used. The presence of the DARPins was confirmed by an antibody recognizing the N-terminal RGS-His tag of the DARPins. As controls, the unselected library member E3_5 and beads with no DARPins were included. The cell lysate used for the pull-down experiments was also analyzed directly for the presence of different JNK isoforms.

E3_5 or with empty beads no JNK was precipitated, the interactions between the selected DARPins and endogenous JNK isoforms seem to be very specific.

Binding Specificities within Cells. Although the pull-down experiments showed the potential of selected DARPins to bind specifically to different endogenous JNK isoforms, the interactions in living cells might be different compared to those in cell lysates. Thus, we chose the most promising DARPins J1_8_27, J2_3_29, J1/2_2_25, J1/2_2_3 and the control DARPin E3_5 and analyzed their binding specificities within cells using bioluminescence resonance energy transfer (BRET) technology. BRET^2 assays (the superscript denoting the use of GFP^2, a variant of green fluorescent protein) were performed in COS-7 cells as described before[42] with slight modifications. All DARPins were expressed as DARPin-GFP^2 fusions and the kinases JNK1α1, JNK2α2 and the highly similar MAPK ERK2 as *Renilla* luciferase (Rluc) fusions (Rluc-kinase) (Figure 5A). JNK1α1 and JNK2α2 were chosen as they seem to be the most abundant splice forms in cells.[34−37]

The results obtained with BRET^2 assays showed a significantly higher binding signal of J1_8_27 to JNK1 compared to JNK2 and ERK2, confirming JNK1 specificity within cells (Figure 5B). In the case of DARPin J2_3_29, the expected specific interaction with JNK2 was observed, but no interaction with JNK1 or ERK2. DARPins J1/2_2_25 and J1/2_2_3 recognized both JNK1 and JNK2, but not ERK2, demonstrating their specificity for both JNK isoforms also in living cells. All selected DARPins thus showed significantly higher specific binding signals for their corresponding JNK isoforms, compared to the binding signals obtained for control DARPin E3_5, implying that the interactions of the individual DARPins are specific within these cells.

These findings (Figure 5B) are in good agreement with the results obtained from pull-down experiments (Figure 4), confirming that the interactions of the selected DARPins are also isoform-specific for JNK1 and JNK2 within intact cells.

Inhibition Properties in Cells. To identify specific intracellular inhibitors of JNK1 and JNK2, the DARPins were further tested for their inhibitory effect on JNK activation in eukaryotic cells. To achieve inhibition in cells, transfection must be very efficient and the intracellular expression levels of the

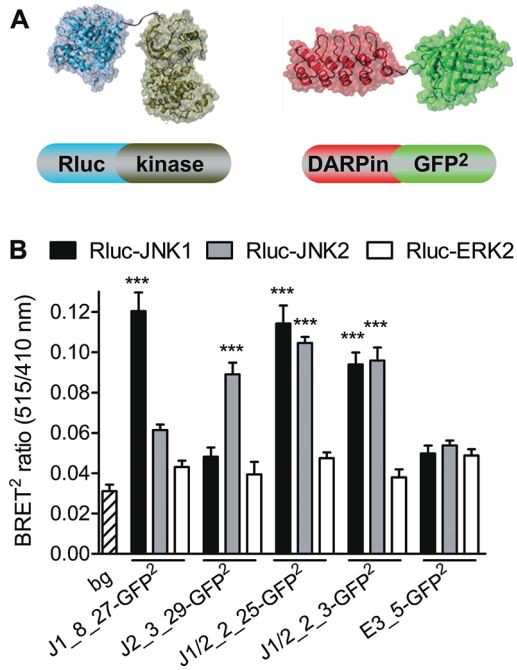

Figure 5. Analysis of kinase specific DARPin interactions in cells. (A) Fusion expression constructs for BRET^2 analyses in living cells. (B) For BRET^2 analyses, COS-7 cells were co-transfected with the plasmids (1:1 DNA ratio) coding for the indicated Rluc-kinase and DARPin-GFP^2 fusion proteins and grown for 24 h. Light emission by *Renilla* luciferase (Rluc) and GFP^2 was followed immediately after incubation with luciferase substrate. For each interaction, six wells were tested. Data shown are mean ± SEM of 3−4 independent experiments. *** indicates a highly significant change ($p > 0.001$) compared to the corresponding DARPin E3_5-GFP^2 data set. bg, Rluc expressed without a fusion partner.

DARPins should be high and exceed the expression levels of endogenous JNK isoforms. In order to obtain a high expression of DARPins in mammalian cells, we optimized the Kozak sequence including the N-terminal tag sequence for detection (FLAG-tag). Since DARPins were originally designed for high-level production in *E. coli*,[20] also the DNA sequences of the DARPins were codon-optimized for mammalian expression and fused to EGFP in order to monitor both the transfection efficiency and expression levels.

To apply a rather fast method to investigate intracellular inhibition, we used a transient transfection method to deliver DARPin genes into cells to achieve constitutive and high intracellular expression levels of the inhibitors. Therefore, HEK 293T cells were transiently transfected with a vector expressing the corresponding DARPin or the empty vector pcDNA3.1 as control, and after expression for 24 h the JNK pathway was activated with anisomycin for 1 h and the cell lysate was analyzed on Western blot. Anisomycin, originally discovered as an antibiotic that blocks translation, activates the JNK pathway already at lower concentrations than needed for inhibition of translation, but the mechanism is not fully understood.[43] All DARPins were expressed at similar levels as judged from the band intensities obtained with anti-FLAG antibody (Figure 6A). The activation of the JNK pathway was detectable with the phospho-JNK-specific antibody only after anisomycin treatment, while the level of total endogenous JNKs remained constant. Two bands were visible with both anti-phospho-JNK antibody and anti-JNK antibody, corresponding to JNK p46

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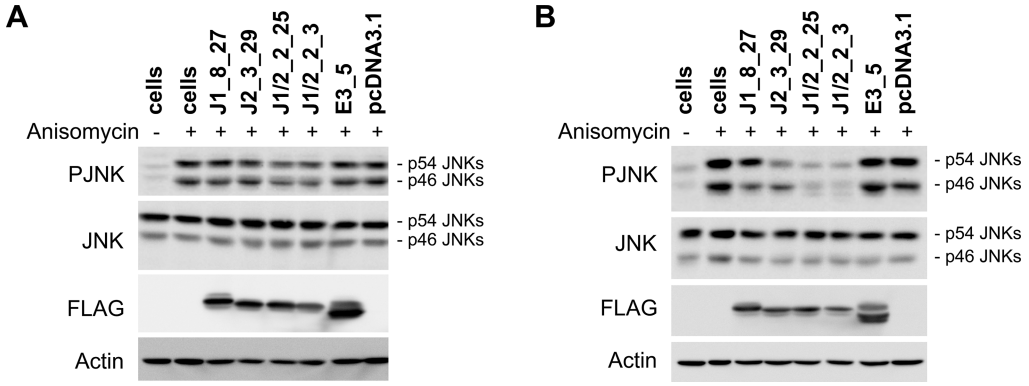

Figure 6. Inhibition properties of selected DARPins in cells. (A) HEK 293T cells were transiently transfected with FLAG-DARPin-EGFP constructs or empty vector pcDNA3.1 as control. Cells were grown for 24 h and then activated with anisomycin for 1 h before the cell lysates were analyzed by Western blotting. Specific antibodies against the phosphorylated (P) JNK isoforms, and all endogenous JNK isoforms were used as well as an antibody recognizing the N-terminal FLAG tag of the DARPins. β-Actin was used as loading control and was detected by a direct antibody against actin. (B) Same as in panel A, but 5% of the most fluorescent cells were sorted by FACS prior to activation with anisomycin to eliminate non-transfected cells and cells with low DARPin expression.

and p54 isoforms. Yet, in the first set of experiments, only a small reduction of the phosphorylated JNK isoforms was observed in the presence of DARPins J1/2_25 and J1/2_2_3, while J1_8_27 and J2_3_29 even showed no detectable effect (Figure 6A and Supplementary Figure S5B). The reason for this incomplete inhibition was found to be the high JNK background activity derived from non-transfected cells and the insufficient inhibition by the low DARPin-expressing cells as a result of inefficient transfection: when analyzing the transiently transfected cells by flow cytometry, a cell population corresponding to non-transfected cells was found, and even the transfected cells showed variations in DARPin expression levels for each tested construct (Supplementary Figure S5A). We thus needed to enrich the high DARPin-expressing cells (see below).

To further rule out a stimulating effect of the expressed DARPins themselves on JNK pathway activation, transiently transfected cells were analyzed for activated JNK isoforms before and after anisomycin treatment. The expression of DARPins by itself did not initiate JNK phosphorylation, since JNK-phospho-specific bands were detected only after addition of anisomycin (Supplementary Figure S5B). Hence, we concluded that the observed poor inhibitory effect was mainly due to the analysis of a mixed cell population, including cells with no or insufficient DARPin expression resulting from low transfection efficiency.

We therefore modified the experimental setup to include a pre-sorting step with fluorescence-activated cell sorting (FACS) to eliminate non-transfected cells and cells with low DARPin expression levels. Cells were transiently transfected with DARPin constructs and after 24 h of expression 5% of the most fluorescent cells were collected by FACS for each DARPin tested. The same cell numbers were also collected for non-transfected cells and cells transfected with pcDNA3.1 vector as controls. After sorting, the cells were activated with anisomycin and cell lysates were analyzed by Western blotting as described before. Under these conditions, overexpression of DARPins J1/2_25 and J1/2_2_3 resulted in almost complete inhibition of JNK activation in the sorted cells, since both bands corresponding to phosphorylated JNKs were hardly detectable (Figure 6B). Interestingly, in the presence of DARPin J1_8_27, a significant reduction of phosphorylated p46 isoforms was visible, while p54 phosphorylation did not seem to be affected. On the other hand, DARPin J2_3_29 showed stronger inhibitory effect on the p54 isoforms compared to the p46 isoforms. As expected, control DARPin E3_5 and the vector pcDNA3.1 did not affect JNK phosphorylation (Figure 6B).

Since the phospho-specific antibody does not discriminate between JNK1 and JNK2 isoforms, we cannot draw any direct conclusions on specificity toward JNK1 and JNK2 from these inhibition experiments. Nevertheless, most cells that are reported express mainly the short p46 kDa splice variant of JNK1 and the long p54 kDa splice variant of JNK2.[34−37] If this is also true for HEK 293T cells, we can postulate that the JNK1-specific DARPin J1_8_27 lowers mainly JNK1 activation, whereas the JNK2-specific DARPin J2_3_29 inhibits mainly JNK2. DARPins J1/2_25 and J1/2_2_3, which recognize both JNK1 and JNK2, led to the inhibition of both the long and the short splice forms, thus inhibiting both JNK species. This interpretation would be in good agreement with the binding specificities of the DARPins observed *in vitro* and in cells as well as with the specific inhibition found *in vitro*.

We thus demonstrated that the generated DARPins can be very potent intracellular inhibitors of the JNK pathway when expressed at high levels in all cells acting at the level of JNK activation. Furthermore, there is compelling evidence that these DARPins are indeed able of isoform-specific inhibition of JNK1 and JNK2 in cells.

In order to apply DARPins intracellularly for extended biological studies, the delivery system has to be further improved. Since DARPins are folded proteins, they cannot cross the cell membrane and thus cannot be applied directly to the cells. Adenoviral gene transfer could provide an interesting solution, as it is a very powerful tool to efficiently introduce foreign DNA into the tissue of interest, combined with transient expression. In addition, adenovirus (Ad)-based vectors have the ability to transduce a wide variety of cell types in a cell-cycle-independent fashion.[44] Adenoviral gene transfer of the JNK binding domain of JNK-interacting protein-1 (JIP-1) was already successfully used for the inhibition of the JNK pathway in mice.[45] To make the Ad-based transgene delivery to desired cells more efficient and target-specific, the native tropism of the virus has to be altered. In our lab we generated specific DARPins against the surface Ad fiber protein as adapters and fused them with a DARPin specific for Her2, a

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cell-surface biomarker of cancer cells.[46] This approach led to the specific transduction of Her2-overexpressing cells in contrast to control cells, confirming the effective targeting of the viral delivery to the desired target cells. We envision that the selected JNK isoform-specific DARPins could be combined with cell-specific Ad-based gene delivery to inhibit the JNK pathway specifically in pancreatic cells, heart or liver to target diabetes and heart and liver diseases where individual JNK isoforms were shown to be involved.[8]

Intracellular DARPins with isoform specificity might thus help guide the development of small molecules toward the relevant epitopes, most importantly when there are no specific small molecule lead candidates available. The use of crystal structures of JNK-DARPin complexes could serve as a starting point for structural-based development of small molecule drugs with the same inhibition mode. Whether DARPins will themselves become a promising alternative to small molecules and peptide inhibitors will depend on the further development of the delivery system.

METHODS

Cloning and Production of Recombinant MAPK Proteins. Human JNK1α1 (pAT222_huJNK1α1, previously published as pAT222_JNK1), human JNK2α2 (pAT222_huJNK2α2, previously published as pAT222_JNK2) and human JNK2α1 (pAT222_huJNK-2α1) fused to pD (avi_pD_JNKisoform_His$_6$) were produced in non-phosphorylated forms in *E. coli* as described previously[27,41] with some modifications as described in the Supporting Information. Human JNK1α1, human JNK2α1, rat ERK2 and mouse p38α without pD fusion partner were produced in non-phosphorylated and phosphorylated forms as described elsewhere.[40] All protein masses were confirmed by SDS-PAGE and mass spectrometry.

***In Vitro* Selections of JNK-Specific DARPins with Ribosome Display.** Ribosome display selections rounds were carried out on plates as previously described[25,27] with some adaptations. For details, see the Supporting Information.

Production of Selected Binders. Selected DARPins were subcloned into pQE30-derived vector (pQE30ss,[47] containing an N-terminal MRGS(His)$_6$-tag) and produced in *E. coli* as described previously[20,29] and in the Supporting Information. The unselected DARPin E3_5 was produced from pQIss_E3_5 (kindly provided by A. Mohr) and DARPin J1/2_2_3 (original name JNK2_2_3) from pQE30_JNK2_2_3.[27]

ELISA. Crude extract ELISA and quantitative ELISA were performed as described[27,29] with some modifications that can be found in the Supporting Information. All steps were performed at 4 °C due to the instability of some kinases.

Size Exclusion Chromatography (SEC) of DARPins and Multi-Angle (Static) Light Scattering (SEC-MALS) of JNK-DARPin Complexes. All measurements were performed on an Agilent LC1100 HPLC system (Agilent Technologies) combined with a miniDAWN three-angle light-scattering detector and an Optilab rEX differential refractometer (Wyatt Technology). Molar masses were calculated using the ASTRA V software (Wyatt Technology). Details can be found in the Supporting Information.

Surface Plasmon Resonance (SPR) Measurements. All measurements were performed at 15 °C on a ProteOn system (Bio-Rad) with immobilized pD_JNK1α1 and pD_JNK2α1 and DARPins as analytes in solution. The procedure, all sensorgrams and fits can be found in the Supporting Information.

***In Vitro* Inhibition.** *In vitro* ELISA-based kinase assays were performed as described [39] with some adaptations. GST-c-Jun (residues 1−79) substrate[48] was expressed in *E. coli* XL1-Blue and purified over a glutathione affinity column according to the manufacturer's instructions (Pharmacia Biotech).

Due to the instability of the kinases, incubation steps were performed at 4 °C, if not mentioned otherwise, and kinase reactions at 20 °C. All assays were optimized for linearity. Non-absorbent microtiter plates were blocked with blocking buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% (w/v) BSA and 0.05% (v/v) Tween-20) for 1 h. After washing the plate, each DARPin (250 nM) was mixed with either non-phosphorylated, biotinylated pD_JNK1α1 or pD_JNK2α1 (4 nM) in a total volume of 120 μL kinase buffer/well (50 mM Tris pH 7.4, 10 mM $MgCl_2$, 0.01% (w/v) BSA, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM DTT, 1 mM ATP) for 1 h to allow binding. The kinase reaction was started by adding 2 ng of active MKK4 (SignalChem) and continued for 1 h.

To address the rate of JNK phosphorylation (JNK activation), in each case 100 μL of the kinase reaction solution was transferred to a well of a MaxiSorp plate that had been previously coated with neutravidin (66 nM) and blocked with BSA. After 1 h of incubation for capture of the biotinylated kinases, the plate was washed, and an anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody (Cell Signaling, 1:1000) was added for 2 h at 37 °C. As secondary antibody, alkaline phosphatase-labeled goat anti-rabbit IgG (Sigma, 1:3000) was incubated for 1 h at 37 °C. Plates were washed, and substrate solution (3 mM 4-NPP, 50 mM $NaHCO_3$, 50 mM $MgCl_2$) was added for 1 h at 37 °C before the A_{410} signal was measured in a plate reader.

To investigate the rate of c-Jun phosphorylation (JNK kinase activity), 10 μL of the kinase reaction solution was transferred to a well of a MaxiSorp plate that had been previously coated with GST-c-Jun (0.5 μg/well), blocked with BSA and filled with 90 μL of kinase buffer. A second kinase reaction was performed for 1 h, and phosphorylated c-Jun was detected with an anti-phospho-c-Jun antibody (Cell Signaling, 1:1000) for 2 h at 37 °C followed by incubation with the same secondary antibody as described above.

To investigate the direct influence of DARPins on c-Jun phosphorylation, each DARPin (250 nM) was mixed with phosphorylated, biotinylated JNK1α1 or JNK2α1 (1 nM), and 10 μL of this mixture was used directly for the second kinase reaction to phosphorylate c-Jun as described above.

The effect of DARPins on JNK phosphorylation was also investigated with crude extract from UV-activated human embryonal kidney 293T (HEK 293T) cells. Crude extract was prepared as described.[39] The kinase assay was performed as described above, except that the first kinase reaction was initiated with 5 μg of crude extract instead of 2 ng of active MKK4.

Pull Down Experiments. DARPins were coupled to Ni-NTA agarose beads, and pull-down experiments were performed with HEK 293T cells (ATCC) lysed in 50 mM Tris pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 1% (v/v) NP-40 as described in the Supporting Information and ref 41. JNK isoforms and DARPins were verified on Western blots using primary antibodies against JNK1 (F-3) (Santa Cruz Biotechnology, 1:200), against JNK2 (long splice forms) (Cell Signaling, 1:2000) and against the RGS-His-tag (1:5000), followed by corresponding secondary antibodies against rabbit and mouse IgGs, respectively.

BRET^2 Assays. Human JNK1α1, human JNK2α2 and rat ERK2 were fused to the C-terminus of Rluc and the selected DARPins to the N-terminus of GFP^2. Cloning of BRET^2 constructs and optimization can be found in the Supporting Information. COS-7 cells (ATCC CRL-1651) were cultivated in DMEM including 5% (v/v) FBS Gold (PAA) as described before[42] and transfected in white 96-well microplates (Nunc) using 1 μL of polyethylenimine (PEI, 1 mg/mL, Polysciences) and 0.2 μg DNA per plasmid and well. Twenty-four hours after transfection, cells were rinsed with phosphate buffered saline (PBS), and 5 μM Coelenterazine 400a (final concentration) (Biotrend) was added immediately before BRET^2 read-out. The light output of Rluc and GFP^2 were measured simultaneously (read time 1 s) for each well with filters at 410 nm wavelength (±80 nm band-pass) for the Rluc and 515 nm (±30 nm band-pass) for the GFP^2 emission using a POLARstar Omega microplate reader (BMG Labtech). As a control, wells containing cells expressing Rluc alone were included in every experiment (background BRET^2 signal, bg). The emission values from non-transfected (nt) cells were subtracted from the emission values of the transfected (em) cells, and BRET^2 signals were calculated as follows: BRET^2 ratio = $(em_{515\ nm} - nt_{515\ nm})/(em_{410\ nm} - nt_{410\ nm})$. Experiments were repeated at least three times with six wells per

experimental condition. Statistical evaluation (one-way ANOVA with Newman Keuls post tests) was carried out with GraphPad Prism software version 5.01 (GraphPad Software).

Inhibition Experiments in Cells. DARPin genes and Kozak sequence including N-terminal tag were optimized for mammalian expression. The resulting plasmids pcDNA3.1(+)-Bi_DARPin#opt_EGFPdeltaMV contain a certain DARPin with a FLAG-tag at the N-terminus and a linker followed by EGFP at the C-terminus (FLAG-DARPin-$(G_4S)_2$-EGFP). The adherent HEK 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin-streptomycin at 37 °C in a CO_2 incubator. One day before transient transfection, 4×10^5 HEK 293T cells in 1 mL of growth medium were added to each well of a 12-well plate (growth area 4 cm^2). Cells were transfected at 90% confluency with 2 μg of DARPin plasmid DNA and 5 μL of Lipofectamine 2000 (Invitrogen) per well. Medium was changed 5 h after transfection. Cells were incubated at 37 °C in a CO_2 incubator for 24 h to allow expression followed by activation with 2 μg/mL anisomycin (Sigma) for 1 h at 37 °C (a negative control was not treated with anisomycin). Cells were washed once with cold PBS and then lysed in 120 μL of ProteoJET (Fermentas) containing complete, EDTA-free protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail PhosSTOP (Roche) for 10 min at 4 °C followed by a centrifugation step at 11,000 × *g* for 10 min at 4 °C. All samples were adjusted to the same concentration using a BCA protein assay (Pierce).

To obtain only cells with very high DARPin expression level, more cells were transfected and incubated for 24 h as described above. Then the cells were suspended by trypsinization and sorted on a BD FACSAria II cell sorter (BD Biosciences). Five percent of total cells with the highest EGFP fluorescence were sorted at RT, and 800,000 cells were collected in growth medium. As controls, 800,000 non-transfected cells were obtained. After sorting, cells (except the negative control) were activated with 2 μg/mL anisomycin for 1 h at 37 °C. Cells were washed once with PBS and lysed in 50 μL of ProteoJET containing complete, EDTA-free protease inhibitor cocktail and phosphatase inhibitor cocktail PhosSTOP.

For detection, cleared cell lysates were heated to 95 °C and analyzed by Western blotting. All antibodies were diluted in $TBS_{150}T$ buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween-20) containing 2.5% (w/v) milk. Membranes were incubated overnight at 4 °C with the following antibodies: anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody (Cell Signaling, 1:1000); anti-SAPK/JNK(56G8) antibody (Cell Signaling, 1:1000); anti-FLAG M2 antibody (Sigma, 1:10,000). The corresponding secondary antibodies were applied for 1.5 h at RT: horse radish peroxidase (HRP)-linked anti-rabbit IgG antibody (Cell Signaling, 1:3000); HRP-linked anti-mouse IgG antibody (Pierce, 1:10,000). As a loading control, actin was detected directly with HRP-linked anti-beta-actin antibody (Sigma, 1:10,000) for 1.5 h at RT. Lumiglo reagent and Peroxide (Cell Signaling) were used for visualization by enhanced chemiluminescence.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev. 7*, 2135−2148.

(2) Boulton, T. G., and Cobb, M. H. (1991) Identification of multiple extracellular signal-regulated kinases (ERKs) with antipeptide antibodies. *Cell Regul. 2*, 357−371.

(3) Han, J., Lee, J. D., Bibbs, L., and Ulevitch, R. J. (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science 265*, 808−811.

(4) Zhou, G., Bao, Z. Q., and Dixon, J. E. (1995) Components of a new human protein kinase signal transduction pathway. *J. Biol. Chem. 270*, 12665−12669.

(5) Kyriakis, J. M., and Avruch, J. (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev. 81*, 807−869.

(6) Chen, Z., Gibson, T. B., Robinson, F., Silvestro, L., Pearson, G., Xu, B., Wright, A., Vanderbilt, C., and Cobb, M. H. (2001) MAP kinases. *Chem. Rev. 101*, 2449−2476.

(7) Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B., and Davis, R. J. (1996) Selective interaction of JNK protein kinase isoforms with transcription factors. *EMBO J. 15*, 2760−2770.

(8) Bogoyevitch, M. A. (2006) The isoform-specific functions of the c-Jun N-terminal Kinases (JNKs): differences revealed by gene targeting. *BioEssays 28*, 923−934.

(9) Bogoyevitch, M. A., and Arthur, P. G. (2008) Inhibitors of c-Jun N-terminal kinases: JuNK no more? *Biochim. Biophys. Acta 1784*, 76−93.

(10) Bogoyevitch, M. A., Ngoei, K. R., Zhao, T. T., Yeap, Y. Y., and Ng, D. C. (2010) c-Jun N-terminal kinase (JNK) signaling: recent advances and challenges. *Biochim. Biophys. Acta 1804*, 463−475.

(11) Bennett, B. L., Sasaki, D. T., Murray, B. W., O'Leary, E. C., Sakata, S. T., Xu, W., Leisten, J. C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S. S., Manning, A. M., and Anderson, D. W. (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl. Acad. Sci. U.S.A. 98*, 13681−13686.

(12) Bogoyevitch, M. A. (2005) Therapeutic promise of JNK ATP-noncompetitive inhibitors. *Trends Mol. Med. 11*, 232−239.

(13) Ngoei, K. R., Catimel, B., Church, N., Lio, D. S., Dogovski, C., Perugini, M. A., Watt, P. M., Cheng, H. C., Ng, D. C., and Bogoyevitch, M. A. (2011) Characterization of a novel JNK (c-Jun N-terminal kinase) inhibitory peptide. *Biochem. J. 434*, 399−413.

(14) Wörn, A., and Plückthun, A. (2001) Stability engineering of antibody single-chain Fv fragments. *J. Mol. Biol. 305*, 989−1010.

(15) Philibert, P., Stoessel, A., Wang, W., Sibler, A. P., Bec, N., Larroque, C., Saven, J. G., Courtete, J., Weiss, E., and Martineau, P. (2007) A focused antibody library for selecting scFvs expressed at high levels in the cytoplasm. *BMC Biotechnol. 7*, 81.

(16) Perez-Martinez, D., Tanaka, T., and Rabbitts, T. H. (2010) Intracellular antibodies and cancer: new technologies offer therapeutic opportunities. *BioEssays 32*, 589−598.

(17) Harmsen, M. M., and De Haard, H. J. (2007) Properties, production, and applications of camelid single-domain antibody fragments. *Appl. Microbiol. Biotechnol. 77*, 13−22.

(18) Gilbreth, R. N., Truong, K., Madu, I., Koide, A., Wojcik, J. B., Li, N. S., Piccirilli, J. A., Chen, Y., and Koide, S. (2011) Isoform-specific monobody inhibitors of small ubiquitin-related modifiers engineered

using structure-guided library design. *Proc. Natl. Acad. Sci. U.S.A. 108*, 7751−7756.
(19) Wojcik, J., Hantschel, O., Grebien, F., Kaupe, I., Bennett, K. L., Barkinge, J., Jones, R. B., Koide, A., Superti-Furga, G., and Koide, S. (2010) A potent and highly specific FN3 monobody inhibitor of the Abl SH2 domain. *Nat. Struct. Mol. Biol. 17*, 519−527.
(20) Binz, H. K., Stumpp, M. T., Forrer, P., Amstutz, P., and Plückthun, A. (2003) Designing repeat proteins: well-expressed, soluble and stable proteins from combinatorial libraries of consensus ankyrin repeat proteins. *J. Mol. Biol. 332*, 489−503.
(21) Boersma, Y. L., and Plückthun, A. (2011) DARPins and other repeat protein scaffolds: advances in engineering and applications. *Curr. Opin. Biotechnol. 22*, 849−857.
(22) Bork, P. (1993) Hundreds of ankyrin-like repeats in functionally diverse proteins: mobile modules that cross phyla horizontally? *Proteins 17*, 363−374.
(23) Canepa, E. T., Scassa, M. E., Ceruti, J. M., Marazita, M. C., Carcagno, A. L., Sirkin, P. F., and Ogara, M. F. (2007) INK4 proteins, a family of mammalian CDK inhibitors with novel biological functions. *IUBMB Life 59*, 419−426.
(24) Forrer, P., Stumpp, M. T., Binz, H. K., and Plückthun, A. (2003) A novel strategy to design binding molecules harnessing the modular nature of repeat proteins. *FEBS Lett. 539*, 2−6.
(25) Hanes, J., and Plückthun, A. (1997) In vitro selection and evolution of functional proteins by using ribosome display. *Proc. Natl. Acad. Sci. U.S.A. 94*, 4937−4942.
(26) Steiner, D., Forrer, P., Stumpp, M. T., and Plückthun, A. (2006) Signal sequences directing cotranslational translocation expand the range of proteins amenable to phage display. *Nat. Biotechnol. 24*, 823−831.
(27) Binz, H. K., Amstutz, P., Kohl, A., Stumpp, M. T., Briand, C., Forrer, P., Grütter, M. G., and Plückthun, A. (2004) High-affinity binders selected from designed ankyrin repeat protein libraries. *Nat. Biotechnol. 22*, 575−582.
(28) Zahnd, C., Pecorari, F., Straumann, N., Wyler, E., and Plückthun, A. (2006) Selection and characterization of Her2 binding-designed ankyrin repeat proteins. *J. Biol. Chem. 281*, 35167−35175.
(29) Steiner, D., Forrer, P., and Plückthun, A. (2008) Efficient selection of DARPins with sub-nanomolar affinities using SRP phage display. *J. Mol. Biol. 382*, 1211−1227.
(30) Stefan, N., Martin-Killias, P., Wyss-Stoeckle, S., Honegger, A., Zangemeister-Wittke, U., and Plückthun, A. (2011) DARPins recognizing the tumor-associated antigen EpCAM selected by phage and ribosome display and engineered for multivalency. *J. Mol. Biol. 413*, 826−843.
(31) Amstutz, P., Binz, H. K., Parizek, P., Stumpp, M. T., Kohl, A., Grütter, M. G., Forrer, P., and Plückthun, A. (2005) Intracellular kinase inhibitors selected from combinatorial libraries of designed ankyrin repeat proteins. *J. Biol. Chem. 280*, 24715−24722.
(32) Kohl, A., Amstutz, P., Parizek, P., Binz, H. K., Briand, C., Capitani, G., Forrer, P., Plückthun, A., and Grütter, M. G. (2005) Allosteric inhibition of aminoglycoside phosphotransferase by a designed ankyrin repeat protein. *Structure 13*, 1131−1141.
(33) Wyler, E., Kaminska, M., Coic, Y. M., Baleux, F., Veron, M., and Agou, F. (2007) Inhibition of NF-kappaB activation with designed ankyrin-repeat proteins targeting the ubiquitin-binding/oligomerization domain of NEM. *Protein Sci. 16*, 2013−2022.
(34) Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. (2000) Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science 288*, 870−874.
(35) Finch, A., Davis, W., Carter, W. G., and Saklatvala, J. (2001) Analysis of mitogen-activated protein kinase pathways used by interleukin 1 in tissues in vivo: activation of hepatic c-Jun N-terminal kinases 1 and 2, and mitogen-activated protein kinase kinases 4 and 7. *Biochem. J. 353*, 275−281.
(36) Altan, Z. M., and Fenteany, G. (2004) c-Jun N-terminal kinase regulates lamellipodial protrusion and cell sheet migration during epithelial wound closure by a gene expression-independent mechanism. *Biochem. Biophys. Res. Commun. 322*, 56−67.
(37) Dreskin, S. C., Thomas, G. W., Dale, S. N., and Heasley, L. E. (2001) Isoforms of Jun kinase are differentially expressed and activated in human monocyte/macrophage (THP-1) cells. *J. Immunol. 166*, 5646−5653.
(38) Forrer, P., and Jaussi, R. (1998) High-level expression of soluble heterologous proteins in the cytoplasm of *Escherichia coli* by fusion to the bacteriophage lambda head protein D. *Gene 224*, 45−52.
(39) Forrer, P., Tamaskovic, R., and Jaussi, R. (1998) Enzyme-linked immunosorbent assay for measurement of JNK, ERK, and p38 kinase activities. *Biol. Chem. 379*, 1101−1111.
(40) Kummer, L., Parizek, P., Rube, P., Millgramm, B., Prinz, A., Mittl, P. R. E., Kaufholz, M., Zimmermann, B., Herberg, F. W., and Plückthun, A. (2012) Phosphorylation-specific ERK kinase-binders from designed ankyrin repeat protein libraries. *Proc. Natl. Acad. Sci. U.S.A.*,in press.
(41) Amstutz, P., Koch, H., Binz, H. K., Deuber, S. A., and Plückthun, A. (2006) Rapid selection of specific MAP kinase-binders from designed ankyrin repeat protein libraries. *Protein Eng. Des. Sel. 19*, 219−229.
(42) Prinz, A., Diskar, M., Erlbruch, A., and Herberg, F. W. (2006)) Novel, isotype-specific sensors for protein kinase A subunit interaction based on bioluminescence resonance energy transfer (BRET). *Cell. Signalling 18*, 1616−1625.
(43) Cano, E., Doza, Y. N., Ben-Levy, R., Cohen, P., and Mahadevan, L. C. (1996) Identification of anisomycin-activated kinases p45 and p55 in murine cells as MAPKAP kinase-2. *Oncogene 12*, 805−812.
(44) Amalfitano, A., and Parks, R. J. (2002) Separating fact from fiction: assessing the potential of modified adenovirus vectors for use in human gene therapy. *Curr. Gene Ther. 2*, 111−133.
(45) Xia, X. G., Harding, T., Weller, M., Bieneman, A., Uney, J. B., and Schulz, J. B. (2001) Gene transfer of the JNK interacting protein-1 protects dopaminergic neurons in the MPTP model of Parkinson's disease. *Proc. Natl. Acad. Sci. U.S.A. 98*, 10433−10438.
(46) Dreier, B., Mikheeva, G., Belousova, N., Parizek, P., Boczek, E., Jelesarov, I., Forrer, P., Plückthun, A., and Krasnykh, V. (2011) Her2-specific multivalent adapters confer designed tropism to adenovirus for gene targeting. *J. Mol. Biol. 405*, 410−426.
(47) Huber, T., Steiner, D., Röthlisberger, D., and Plückthun, A. (2007) In vitro selection and characterization of DARPins and Fab fragments for the co-crystallization of membrane proteins: The Na(+)-citrate symporter CitS as an example. *J. Struct. Biol. 159*, 206−221.
(48) Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell 76*, 1025−1037.