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Peptide-DNA conjugates as tailored bivalent binders of the oncoprotein c-Jun

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We describe a ds-oligonucleotide-peptide conjugate that is able to efficiently dismount preformed DNA complexes of the bZIP regions of oncoproteins c-Fos and c-Jun (AP-1), and therefore might be useful as disrupters of AP-1-mediated gene expression pathways.

Introduction
Gene expression is a highly regulated process that involves the interaction of proteins called transcription factors (TFs) with specific regulatory sites of the genome. The dysfunctional activity of TFs can lead to errors in the expression of specific gene products, and eventually to anomalous behaviour of the cells. When TFs are involved in the regulation of cell proliferation events, such as in the case the oncoprotein c-Jun, this irregular behaviour might result in uncontrolled cell proliferation and cancer. In fact, there is a large number of evidences on the presence of unusual levels of c-Jun in different types of human cancers, which suggest that this protein plays a role in the malignant transformation of cells. Therefore, there is a high interest on the development of strategies that enable to control the activity of this transcription factor.

The mechanism of action of c-Jun involves the formation of a coiled-coil heterodimer with other bZIP protein components, prominently with c-Fos, to form the so-called AP-1 complex with specific DNA sites (5'-ATGACTCAT-3'). Earlier reports using this heterodimerization complex as reference, have shown that it is possible to trap c-Jun into an inactive complex by using recombinant proteins featuring the leucine zipper region of c-Fos. This strategy was more effective when the leucine zipper region of c-Fos is modified with an acidic tail designed to increase the affinity by introducing stabilizing electrostatic interactions with the (positively charged) basic region of c-Jun. Therefore, the specificity of these systems relies exclusively on the leucine zipper component of the sequestering agent, which is complementary to that of c-Jun.

We have previously reported oligonucleotide-peptide conjugates that are also capable of interacting with the oncoprotein c-Jun. These systems presented a bivalent surface for the specific simultaneous recognition of both the leucine rich, and the basic region of c-Jun. Unfortunately, although these designs were able to trap the bZIP region of Jun with good affinity at 4 °C, the interaction was relatively weak and independent of the oligonucleotide sequence. Therefore, the conjugates worked as the previously described dominant negative peptides, in which the oligonucleotide unit is just providing a negatively charged surface to favour contacts with the basic region of c-Jun. Moreover, these oligonucleotide-peptide conjugates were not able to disrupt preformed AP-1/DNA complexes.

We have now redesigned the system, and are pleased to report the discovery of a hybrid that binds the bZIP domain of c-Jun at room temperature with high affinity. The interaction of c-Jun with the designed conjugate involves specific contacts of both the leucine zipper region and the oligonucleotidic unit. Importantly, the optimized system allows disassembling of a preformed AP-1 complex.

Results and Discussion

Design an optimization of the dsDNA-peptide conjugates
Our previous constructs consisted of a 35 amino acid sequence of c-Fos (residues 158-192) connected through the N terminus to a 5'-thiol terminated oligonucleotide fragment, and featuring a four-base separation between the 5’ end and the consensus binding sequence of c-Jun (TCAT). As commented before, gel shift experiments demonstrated that the interaction of the conjugates with c-Jun at 4 °C was independent of the dsDNA sequence, suggesting that the basic region of c-Jun interacts with the polyphosphate surface in a nonspecific manner, instead of being inserted in the DNA groove forming specific contacts with the edges of the bases, as happens in the natural AP-1 complex. A qualitative model based on the X-ray structure of the DNA/AP-1 complex suggested that the inability to form specific contacts could arise from an inappropriate arrangement of the c-Jun DNA binding site, which would not allow a strain-less insertion of the c-Jun basic region into the DNA major groove. We envisioned that having a five or even six-base pair spacer between the consensus binding sequence of c-Jun and the 5’ end of the DNA might increase the flexibility of the system, thereby allowing the insertion of the basic region into the groove (Fig. 1). On the other hand, since our previous double stranded oligonucleotide-peptide conjugates also underwent partial dehybridization in the binding assays, we decided to use slightly longer oligonucleotide chains to increase the thermal stability.

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Peptide 1 that contains the leucine-rich region of c-Fos and incorporates a maleimide moiety at the N-terminus, was synthesized on a Rink-4-methyl-benzhydrylamine resin following standard Fmoc/tert-Butyl protocols. The N-terminal maleimide unit was introduced in a final coupling step by using N-maleoyl-β-alanine. Side-chain deprotection and cleavage from the resin (TFA/Et3SiH/H2O, 95:3:2) afforded the desired peptide in a reasonable yield (approximately 12%) and with high purity, as deduced from RP-HPLC analysis. Miniproteins Fos and Jun, which feature the b-ZIP (basic and leucine rich) regions of the natural proteins c-Fos and c-Jun, were obtained by solid phase synthesis, using a PAL-PEG-PS resin following standard procedures. Side-chain deprotection and cleavage from the resin (TFA/EDT/H2O/TIS, 94:2.5:2.5:1) afforded the required peptides.

The coupling reactions between 5′-thiol-terminated oligonucleotides and the maleimide-equipped peptide were carried out by stirring the oligonucleotides (50 µM) with a thiol excess of the peptide at room temperature (rt), in an aqueous buffer containing 10 mM Tris-HCl and 100 mM NaCl at pH 7.5 (Scheme 1). RP-HPLC monitoring of the reaction revealed the relatively rapid formation of new products that had the chromatographic and spectroscopic characteristics expected for the oligonucleotide–peptide conjugates (λmax: 220 and 260 nm, full conversion after 30 min). After HPLC purification, the identity of the products was confirmed by MALDI-TOF MS (see the ESI). The annealing of all conjugates with their corresponding complementary DNA strands was achieved by mixing equimolecular amounts of the conjugates with their complementary oligonucleotides in phosphate buffer 10 mM, NaCl 100 mM, pH 7.5. The double stranded derivatives will be named with the suffix ds.

### Scheme 1 Reaction sequence for the synthesis of oligonucleotide-peptide conjugates, exemplified for the case of conjugate C1. The consensus binding site for c-Jun is shown in bold.

In addition to C1, which includes a four-base pair distance between the consensus binding sequence of c-Jun and the 5′ end of the DNA, we also prepared C2 and C3, which have respectively one and two more spacing bases. We also synthesized C4, which lacks the c-Jun binding sequence, and conjugates with single mutations in the recognition sequence (C2GST and C2CTT) as controls for specificity.

### Scheme 2 Sequences of the synthesized ss-oligonucleotide-peptide conjugates

**Interaction assays of Jun with the oligonucleotide-peptide conjugates**

With the ds-oligonucleotide–peptide conjugates at hand, we were in position to run preliminary binding assays with Jun, which contains the bZIP functional region of the natural protein c-Jun. The interaction was studied by electrophoretic mobility shift assays (EMSA) under non-denaturing conditions. As shown in the gel-shift results (Fig 2a), we observed the formation of retarded bands when Jun was added to the double-stranded conjugates C1ds, C2ds, C3ds. Interestingly, we observed qualitative differences in the binding properties of the three conjugates, with the ones featuring a five or a six base pair spacer between the edge of the DNA and the target sequence showing a better affinity. In particular, C2ds, which contains a 5-base pair spacing, is the one presenting the best properties in terms of affinity. This result confirmed our initial hypothesis that the spacer between the 5′ end of the DNA and the consensus-binding site of c-Jun has an important influence in the formation of the complex.

We next ran several control binding assays in order to study the specificity of the interaction. Importantly, a conjugate that features a dsDNA in which the consensus c-Jun binding sequence has been fully mutated (C4ds) failed to elicit retarded bands when treated with the same concentrations of Jun than in the experiments above (Fig 2b, lanes 2-4). Moreover, even the conjugates in which the oligonucleotide sequence exhibits a single mutation in the consensus recognition site (C2GSTds and C2CTTds) were also unable to show significant Jun trapping.
ability (Fig 2b, lanes 5-10).

A titration with Jun using C2ds containing a 32P radioactively labelled strand, revealed a dissociation constant of ≈ 201 nM at room temperature (see Fig. S1 and S2 in the ESI). To obtain a more quantitative characterization of the recognition process, we also performed fluorescence anisotropy titrations. Thus, incubation of a fluorescein-labelled C2ds with increasing amounts of Jun resulted in a progressive increase of the anisotropy, which was consistent with the formation of a complex with higher molecular weight and reduced mobility. Fitting the resulting data to a 1:1 binding model allowed to calculate its dissociation constant (Kd ≈ 362 nM) at 22ºC. 

A qualitative assessment of structural changes associated to the formation of the complex between the conjugate C2ds and Jun was obtained by circular dichroism. Therefore, while C2ds and the protein Jun are poorly structured as isolated entities (Fig. 3 right, dashed lines), mixing them in equimolar amounts produces an increase in the negative ellipticity of the bands at 208 and 222 nm that correlates with an increase of α-helical content of these species, as expected for the formation of the complex. Moreover, the CD spectrum also indicates a small increase in the intensity of the negative peak at 275 nm upon addition of Jun, which suggests that there is some degree of unwinding in the DNA structure.

With the conjugate C2ds at hand, we were in position to assess whether it could interfere with or even destroy the AP-1/DNA complex. Toward this end, a 32P-labelled double stranded (ds) oligonucleotide containing the AP-1 binding site was incubated with equimolecular amounts of Fos and Jun to give the expected complex as deduced from the formation of a retarded EMSA band (Fig 4, lane 2). Gratifyingly, incubation of this mixture with increasing amounts of the ds conjugate C2ds promoted the progressive disappearance of the slower migrating band. A titration allowed us to calculate an IC50 of ≈ 491 nM (see Fig. S3 in the ESI).

Therefore, this result demonstrates that our ds-oligonucleotide-peptide conjugate C2ds is not only capable of binding free Jun, but it can also disrupt preformed AP-1-like complexes.

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Conclusions

In summary, our data demonstrate that an appropriately designed peptide-oligonucleotide conjugate that present both a DNA and a peptidic recognition surface can bind transcription factors purchased from peptide2oligonucleotide conjugate that present both a DNA and a piperidine/toluene/DMF (2:1:7, 10 mL), opportunities to exploring relevant biomolecular recognition processes, as well as to developing new types of conditional decoy platforms for specific sequestration of transcription factors disrupt their DNA complexes. This strategy offers unique fragment by means of a specific, bivalent interaction, and even like cancers.

Experimental Section

Peptide synthesis

All peptide synthesis reagents and amino acid derivatives were purchased from GL Biochem (Shanghai) and NovaBiochem; standard amino acids were purchased as protected Fmoc amino acids with the standard side chain protecting scheme. C-terminal amide peptides were synthesized using a 0.66 mmol/g loading Rink-MBHA amide resin from GL Biochem (Shanghai) or a 0.21 mmol/g loading Fmoc–PAL–PEG–PS resin from Applied Biosystems. All other chemicals were purchased from Sigma–Aldrich. All solvents were dry and synthesis grade, except DMF for peptide synthesis.

The synthesis of peptide 1 was carried out manually on a Rink-MBHA amide resin (0.66 mmol/g, 0.33 mmol scale) using the following protocol: 1) DMF washings, 5 × 1 min; 2) piperidine/toluene/ DMF (2:1:7, 10 mL), 2 × 2 min; 3) DMF washings, 4 × 1 min; 4) CH₂Cl₂ washing, 2 × 1 min; 5) Fmoc-amino acid (8 eq, 2.4 mmol) and DIC (4 eq, 1.2 mmol) in DMF / CH₂Cl₂ (3 mL/0.6 mL), 1 h. The completion of the coupling reaction was monitored using Kaiser and NF-31 tests. If one of the tests was positive, the coupling reaction was repeated with 4 eq of Fmoc-amino acid, HOAt (4 eq, 1.2 mmol) and HBTU (4 eq, 1.2 mmol) and DIEA (8 eq, 2.4 mmol) in DMF (2 mL). After coupling of the last arginine, an aliquot of resin-bound peptide (50 mg, 0.01 mmol scale) was separated and it was reacted with a solution of N-maleoyl-val-alanine (BMPA, 5 eq, 8.5 mg, 0.05 mmol), HOAt (5 eq, 6.8 mg, 0.05 mmol), HBTU (5 eq, 19.0 mg, 0.05 mmol) and DIEA (10 eq, 17 µL, 0.1 mmol) in DMF (600 µL). The cleavage/deprotection step was performed by treatment of the resin-bound peptide with the following mixture: 950 µL of TFA, 30 µL of Et₂Si and 20 µL of H₂O (300 µL of this mixture for 10 mg of resin). After precipitation in Et₂O, the peptide was dissolved in H₂O / 0.1% TFA (500 µL). Reversed-phase HPLC analysis (performed using an Agilent 1100 series Liquid Chromatograph Mass Spectrometer system, Zorbax Eclipse XDB-C18 (5 µm) 4.6 × 50 mm analytical column from Agilent, gradient of 5 to 95% CH₃CN, 0.1% TFA / H₂O, 0.1% TFA over 30 min, detection at 220 nm, Electrospray Ionization Mass Spectrometry (ESI/MS) (performed with an Agilent 1100 Series LC/MSD VL G1956A model in positive scan mode) revealed that conjugate 1 elutes with a retention time of 13.5 min. The purification of the peptide was performed on a Jupiter Proteo 90A (4 µm) 10 × 250 mm reverse-phase column from Phenomenex and the peptide was identified by ESI/MS ([MH]+) calculated for C₁₇H₁₆N₄O₂S₈ 4238.7, found 4133.4 [MH]+; 1060.5 [MH]+; 848.3 [MH]+). Approximate yield for the whole process 12%.

The miniprotein Fos was synthesized using an automatic peptide synthesizer from Protein Technologies PS1 Peptide Synthesizer on a Fmoc–PAL–PEG–PS resin (0.21 mmol/g, 0.05 mmol scale). The amino acids were coupled in 4-fold excess using HBTU as activating agent. Each amino acid was activated for 30 seconds in DMF before being added onto the resin. Peptide bond-forming couplings were conducted for 30 min to 45min. The deprotection of the temporal Fmoc protecting group was performed by treating the resin with 20% piperidine in DMF solution for 10 min. The cleavage/deprotection step was performed by treatment of the resin-bound peptide (0.02 mmol) with 3 mL of the cleavage cocktail (75 µL of 1.2-ethanediethyl (EDT)), 75 µL of triisopropylsilane (TIS) and TFA to 3 mL) for 3 h. After precipitation in Et₂O, the peptide was redissolved in acetonitrile/water 1:1 (1 mL). Reversed-phase HPLC analysis (same conditions as peptide 1, gradient of 15 to 95% CH₃CN, 0.1% TFA / H₂O, 0.1% TFA over 30 min) revealed that Fos elutes with a retention time of 15.5 min. The purification of the peptide was performed on a Jupiter Proteo 90A (4 µm) 10 × 250 mm reverse-phase column from Phenomenex and the peptide was identified by ESI/MS ([MH]+) calculated for C₁₉H₂₃N₅O₄S₈ 6999.70, found 1383.4 [MH]+; 1152.9 [MH]+; 864.9 [MH]+). Approximate yield for 0.02 mmol scale.

Jun was purchased from Eurogentec (Belgium) attached to a solid support. The final Fmoc deprotection step was carried out using standard conditions. Reversed-phase HPLC analysis (same conditions as Fos) revealed that Jun peptide elutes with a retention time of 16.6 min. The purification was performed as previous described, and the peptide was identified by ESI/MS ([MH]+) calculated for C₁₇H₂₅N₃O₅ 7582.3, found 1265.8 [MH]+; 1087.1 [MH]+; 950.9 [MH]+; 845.5 [MH]+; 760.9 [MH]+).

Synthesis of the oligonucleotide-peptide hybrids

The DNA oligos were purchased from Thermo Fisher Scientific. The conjugation reactions were performed in aqueous buffer solution (10 mM Tris-HCl, 100 mM NaCl, pH 7.5) with thiol-oligo (50 µM) and a threefold excess of the peptide 1 (150 µM). The conjugation reaction was monitored by RP-HPLC (Waters X-Terra column at a flow rate of 1 mL/min, detection at 220 and 260 nm, gradient 0 to 50 % B over 20 min, A: 5% CH₃CN in triethylammonium acetate (TEAA) 100 mM, pH 7.0, B: 70% CH₃CN in TEAA 100 mM, pH 7.0). The oligonucleotide–peptide
conjugates were purified by reversed-phase HPLC and were identified by MS-MALDI-TOF. C1: \( t_{R} = 15.9 \text{ min}, \) [MH] \(^{-}\) calculated for C\(_{359}\)H\(_{535}\)N\(_{113}\)O\(_{78}\)P\(_{3}S\) 9880.12, found 9880.66; C2: \( t_{R} = 15.8 \text{ min}, \) [MH] \(^{-}\) calculated for C\(_{359}\)H\(_{532}\)N\(_{14}\)O\(_{78}\)P\(_{3}S\) 9880.12, found 9879.60; C3: \( t_{R} = 15.9 \text{ min}, \) [MH] \(^{-}\) calculated for C\(_{360}\)H\(_{531}\)N\(_{15}\)O\(_{78}\)P\(_{3}S\) 9920.13, found 9921.50; C4: \( t_{R} = 15.4 \text{ min}, \) [MH] \(^{-}\) calculated for C\(_{360}\)H\(_{532}\)N\(_{15}\)O\(_{78}\)P\(_{3}S\) 9963.16, found 9964.57; C2\(_{CTP}\): \( t_{R} = 15.5 \text{ min}, \) [MH] \(^{-}\) calculated for C\(_{359}\)H\(_{536}\)N\(_{11}\)O\(_{78}\)P\(_{3}S\) 9855.12, found 9854.97; C2\(_{CTP}\): \( t_{R} = 15.6 \text{ min}, \) [MH] \(^{-}\) calculated for C\(_{360}\)H\(_{535}\)N\(_{11}\)O\(_{78}\)P\(_{3}S\) 9895.12, found 9896.05.

### Annealing of the conjugates with complementary oligonucleotides

The oligonucleotide-peptide conjugates were annealed with their complementary DNA strands by using a 15% nondenaturing polyacrylamide gel and 0.5 M Tris·HCl, pH 7.5. The solutions were heated to 90 °C for 10 min and then allowed to slowly cool to room temperature.

#### Binding assays

For gel mobility-shift assays, binding reactions with miniprotein Jun were performed over 30 min, at 22 °C by using ds-oligonucleotide-peptide conjugates (≈ 50 nM) in a binding mixture containing Tris HCl (18 mM, pH 7.5), KCl (90 mM), MgCl\(_{2}\) (1.8 mM), EDTA (1.8 mM), 9% glycerol, bovine serum albumin (0.11 mg/mL) and 2.2% NP-40. Products were resolved by adding increasing amounts of conjugate with a fluorescein-labelled complementary ssDNA, the competition with a fluorescein-labelled complementary ssDNA, and stained with SYBR Gold Nucleic Acid Gel Stain (Molecular Probes) according to the standard protocol.

The titration of Jun was carried out with ≈ 90 pM labelled ds-oligonucleotide-peptide conjugate, constructed by annealing C2 with a 32P-labelled complementary ss-DNA. The competition assays of Fox-Jun complex with conjugate C2ds was carried out with ≈ 90 pM 32P-labelled ds-DNA, Jun ≈ 500 nM and Fox ≈ 500 nM with increasing amounts of conjugate C2ds. The experiments were carried out in the standard binding buffer at 22 °C. The 32P-labelling was carried out using standard kinase methods. Products were resolved by PAGE using a 15% non-denaturing polyacrylamide gel and 0.5× TBE buffer, and analysed by autoradiography.

#### Fluorescence anisotropy

Steady-state fluorescence anisotropy measurements were made in a Jobin-Yvon Fluoromax-3, (DataMax 2.20), coupled to a temperature controller Wavelength Electronics LFI-3751, and using a standard Helma semi-micro cuvette (114F-QS, 10 mm light path). Titrations were carried out at 22 °C using the following parameters: \( \lambda_{ex} = 495 \text{ nm}, \lambda_{em} = 518 \text{ nm}, \) excitation slit width 3.0 nm, emission slit width 12.0 nm, 2 sec integration time, each measure was taken in triplicate. Titrations were made by adding increasing amounts of Jun to a solution containing 50 nM labelled ds-oligonucleotide-peptide conjugate, constructed by annealing C2 with a fluorescein-labelled complementary ssDNA, in 10 mM Tris HCl, pH 7.5.

### Circular Dichroism

Circular dichroism measurements were made with a Jasco J-715 coupled to a Neslab RTE-111 termostated water bath, using a Helma 100-QS macro cuvette (2 mm light path). Measurements were made at 22 °C. Samples contained 10 mM Tris·HCl (pH 7.5), 100 mM NaCl, 5 µM of C2ds ds-oligonucleotide-peptide conjugate and 5 µM of Jun. The spectra are the average of 4 scans and were processed using the “smooth” macro implemented in the program Kaleidograph (v 4.1 by Synergy Software).

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### Notes and references


11 The synthesized models of bZIP proteins Jun and Fos have respectively the following sequences, amino acids 262–324 (Jun): FIKAEKRRMRNRIASAKSRKRKLERSRSERLEEKVTILKAQNSF NASTANLMREQVAQQLQKVMNH; amino acids 138–193 (Fos): MKRRRRERKNMMAKCRNRRRLTDLQAETDQLEDEKSA LQTEIAALLKEKELW.


15 The sequence of the double stranded oligonucleotide used in these experiments is: *ds*-5’-TGGAGATGACTTCATCGT-3’. Indicated in bold the consensus binding site for the heterodimer Fox/Jun.