Synergistic DNA- and Protein-Based Recognition Promote an RNA-Templated Bio-orthogonal Reaction

Niall M. McLoughlin, Arne Kuepper, Saskia Neubacher, and Tom N. Grossmann*
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1. Methods

1.1 Abbreviations

RT: room temperature; Fmoc: fluorenylmethoxycarbonyl; DMF: N,N-dimethylformamide; NMP: N-methyl-2-pyrrolidone; DCM: dichloromethane; PyBOP: benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; COMU: (1-cyano-2-ethoxy-2-oxoethylidenaminoxy)dimethylamino-morpholino-carbenium hexafluorophosphate; HCTU: 2-(6-chloro-1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyleniminium hexafluorophosphate; HATU: 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; Oxyma: ethyl cyano(hydroxyimino)acetate; DIPEA: N,N-diisopropylethylamine; NMM: N-methyl morpholine; Ac₂O: acetic anhydride; AcOH: acetic acid; TFE: 2,2,2-trifluoethanol; TIPS: triisopropylsilane; TFA: trifluoroacetic acid; ACN: acetonitrile; EMSA: Electrophoretic mobility shift assay; TAE: tris-acetate (40 mM tris, 20 mM acetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA)); PBS: phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4);

1.2 Oligonucleotides

The sequences and names of all oligonucleotides used in this study are presented in Supporting Table S1. High-performance liquid chromatography (HPLC)-purified oligonucleotides were purchased from NOXXON Pharma and Sigma-Aldrich. For quantification, the ultraviolet (UV) absorbance of the oligonucleotides was measured in the buffer of the corresponding experiment using a V-550 ultraviolet/visible (UV/Vis) spectrophotometer (Jasco). Respective concentrations were calculated with a molar extinction coefficient at λ = 260 nm, determined according to the nearest-neighbor model using published parameters for oligonucleotides. RNA duplexes were heated to 95 °C for 10 min and slowly cooled to RT for 1 h prior to experiments.

1.3 Solid-phase peptide synthesis

Full details of peptide sequences and final characterization can be found in Supporting Table S2 and Supporting Figures S28, S29 and S33. sTAV2b was synthesized and characterized as previously described. All other peptides were synthesized according to the following procedures on H-Rink amide ChemMatrix® resin (Sigma Aldrich) using a Fmoc-based strategy. General reagents and Fmoc-protected amino acids were purchased from VWR, Carl Roth, Merck, Sigma Aldrich and Thermo Fisher Scientific. 6-Azido-hexanoic acid was purchased from Iris Biotech.

1.3.1 Automated peptide synthesis

Automated peptide synthesis was performed using a Syro II (MultiSynTech). Fmoc-protected amino acids were prepared as 0.33 M solutions dissolved in 0.33 M Oxyma (DMF) and coupling reagents were dissolved in DMF (c = 0.33 M). DIPEA was dissolved in NMP (c = 1.33 M). Fmoc deprotection was carried out in Piperidine/DMF (1/5, v/v), 2 x 5 min. Coupling of amino acids was performed as double couplings,
Fmoc-Xaa-OH (4 eq.), PyBOP/HATU (3.9 eq.) and DIPEA (c = 1.33 M) for 30 min each. After each double coupling cycle, resins were treated with Ac₂O/NMP (1/10, v/v), 2 x 5 min.

1.3.2 Manual peptide synthesis
All reaction steps were performed at room temperature in syringe reactors. Resins were shaken by suspending syringe reactors on an orbital shaker. Synthesis followed a deprotect, couple, cap workflow. Dry resin was typically swollen in DMF for 30 min before an initial reaction. In between reaction steps, resins were washed with DMF (3x, 1 mL per 50 mg of resin), DCM (3x, 1 mL per 50 mg of resin) and DMF (3x, 1 mL per 50 mg of resin).

1.3.3 Fmoc deprotection
Resins were treated with a solution of Piperidine/DMF (1/5, v/v, 1 mL per 50 mg of resin) for 2 x 10 min at RT.

1.3.4 Manual amino acid coupling procedure
Fmoc-Xaa-OH (4 eq.) was prepared with Oxyma (4 eq.) and COMU (4 eq.) in DMF (c = 0.25 M) and activated with DIPEA (8 eq.). The coupling solution was added to the resin and shaken at RT for 30 min. The solution was subsequently discarded, the resin washed and then treated with a second coupling solution composed of Fmoc-Xaa-OH (4 eq.), Oxyma (4 eq.) and PyBOP (4 eq.) in DMF (c = 0.25 M) which was activated with DIPEA (8 eq.). Resins were shaken for 30 min at RT before the coupling solution was discarded.

1.3.5 N-acetylation (Capping)
Free amino groups were acetylated by treating resins with a solution of Ac₂O/DIPEA/DMF (1/1/8, v/v/v, 1 mL per 50 mg of resin) for 2 x 5 min at RT.

1.3.6 MMT deprotection
Resins were swollen in DCM for 30 min prior to treatment with a solution of AcOH/TFE/DCM (1/1/8, v/v/v, 1 mL per 50 mg of resin) for 3 x 20 min at RT.

1.3.7 Bromoacetic acid coupling
Bromoacetic acid (10 eq.) and PyBOP (10 eq.) were dissolved in DMF (c = 0.25 M) and activated with NMM (20 eq.). The coupling solution was then added to the resin and shaken for 2x 120 min at RT.

1.3.8 6-Azido-hexanoic acid coupling
6-azido-hexanoic acid (4 eq.) HCTU, (4 eq.) and Oxyma (4 eq.) were dissolved in DMF (c = 0.25 M) and activated with DIPEA (8 eq.) The coupling solution was then added to the resin and shaken for 2x 30 min at RT.

1.3.9 Final cleavage, purification and characterization
Before final cleavage the resin was dried under vacuum. A solution of TFA/TIPS/H₂O (94/3/3, v/v/v, 2 mL/20 µmol resin) was added to the resin for 4x 1 h. The cleavage solution was then partially evaporated
followed by the addition of cold diethyl ether to precipitate the crude peptide. After centrifugation (4 °C, 4000 rpm, 15 min), the supernatant was discarded, the crude product was dissolved in H2O/ACN (5/1, v/v) and lyophilized. Crude lyophilised peptides were re-dissolved in H2O/ACN (19/1, v/v) and purified by reversed-phase HPLC (Column: Macherey-Nagel Nucleodur C18, 10 × 125 mm, 110 Å, 5 μm. Solvent A: H2O + 0.1 % TFA Solvent B: ACN + 0.1 % TFA. Flow Rate: 6 mL·min⁻¹). An isocratic gradient from 5 – 40% Solvent B over 40 min was typically used for peptide purification. Pure fractions were subsequently pooled and lyophilized followed by characterization and quantification.

Peptides were characterized using an analytical reversed-phase HPLC (1260 Infinity, Agilent Technology. Column: Agilent Eclipse XDB-C18, 4.6x150 mm, 5 μm. Solvent A: H2O + 0.1 % TFA, Solvent B: ACN + 0.1 % TFA. Flow Rate: 1 mL·min⁻¹, 5 – 65 % gradient over 30 min) coupled to an ESI-MS (6120 Quadrupole LC/MS, Agilent Technology). Analytical HPLC chromatograms at 210 nm and MS spectra (masses and m/z ratios in Supporting Table S2) are provided in Supporting Figures S28, S29 and S33. Peptides containing tryptophan were quantified by UV absorption (λ = 280 nm; ε = 5960 M⁻¹·cm⁻¹) in 100 mM sodium phosphate buffer (pH 8.5). Peptides not containing tryptophan were quantified by HPLC-based comparison (210 nm) with reference to a gravimetrically-quantified peptide standard.

1.4 Peptide Truncate Design

To determine adequate peptide truncations, the crystal structure of two TAV2b proteins in complex with double-stranded palindromic siRNA (PDB ID: 2ZI0) was examined using PyMOL (Schrödinger). Here amino acid positions (corresponding to sTAV2b) found to make polar contacts with a given nucleotide backbone were enumerated according to the corresponding oligonucleotide strand. This provided a set of hypothetical contacts between protein and RNA components. Assuming that a similar contact pattern would occur upon binding to an RNA⁹/DNA⁸ duplex, we substituted the sequences of the palindromic RNA duplex for RNA⁶/DNA⁶, yielding Supporting Figures S6 and S7. As we aimed to conjugate 12-mer DNA strands to each conjugate, this matrix allowed us to rationalize the number of potential polar contacts between each conjugate and RNA⁶ for both A and B arrangements.

1.5 Peptide-DNA conjugation, purification and characterization

For conjugation reactions, disulfide-protected, 5’ or 3’, thiolated DNA (Sigma-Aldrich) were prepared as 2 mM stock solutions in nuclease free water. DNA stocks were then diluted in conjugation buffer (0.75 M ammonium bicarbonate, pH 8.0) with 200 mM TCEP (intermediate concentrations: c = 0.66 mM DNA, c = 2.64 mM TCEP (4 eq.)) and shaken at RT for 1 hour. Subsequently, 12.5 mM bromoacetamide-labelled peptide stock was added to the reaction solution (final concentrations: c = 0.5 mM DNA, c = 2 mM TCEP (4 eq.), c = 3 mM peptide (6 eq.)) and incubated for 90 min at room temperature. After peptide addition, conjugation reactions were monitored by analytical reversed-phase HPLC (1100, Agilent Technology. Column: Agilent AdvancedBio Oligonucleotides, 4.6 x 150 mm, 2.7 μm. Solvent A: H2O +0.1 % TFA, Solvent B: ACN + 0.1% TFA. Flow Rate: 1 mL·min⁻¹). Upon reaction completion, an isocratic gradient
from 10-40% solvent B over 40 min was used for conjugate purification. In the case of 2-B11 and 2-B10, conjugation reactions were monitored using the same instrumentation but with a different solvent system (Solvent A: 0.5 M TEAA, pH 8, Solvent B: ACN). For 2-B11 and 2-B10 purification, an isocratic gradient from 5-25% solvent B over 20 min was used. Pure fractions were subsequently pooled and lyophilized. Dry stocks were re-dissolved in nuclease free water and buffer exchanged using an Amicon® 3kDa ultracentrifugal filter (Merck) for follow-up experiments.

Conjugate purity was assessed using an analytical RP-HPLC (1100, Agilent Technology). Column: Agilent AdvancedBio Oligonucleotides, 4.6 x 150 mm, 2.7 µm. Solvent A: H₂O +0.1 % TFA, Solvent B: ACN + 0.1% TFA. Flow Rate: 1 mL·min⁻¹, 10 – 50 % gradient over 10 min). In the case of 2-B11 and 2-B10, purity was assessed using the same instrumentation but with a different solvent system (Solvent A: 0.5 M TEAA, pH 8, Solvent B: ACN, Flow Rate: 1 mL·min⁻¹, 10 – 25 % gradient over 10 min). Corresponding chromatograms are provided in Supporting Figures S30 and S31. Mass spectra of conjugates were obtained using an analytical reversed-phase HPLC (1260 Infinity, Agilent Technology). Column: Agilent InfinityLab Poroshell 120 EC-C18, 2.1 x 50 mm, 2.7 μm. Solvent A: H₂O + 0.1% TFA, Solvent B: ACN + 0.1% TFA. Flow Rate: 0.6 mL·min⁻¹) coupled to an ESI-MS (6120 Quadrupole LC/MS, Agilent Technology). In the case of 2-B11 and 2-B10, mass spectra were collected using a MALDI-TOF MS (Bruker ultrafleXtreme™). For MALDI analysis, conjugate samples were diluted in freshly prepared matrix solution (400 mM 3-hydroxypicolinic acid, 25 mM ammonium citrate dibasic in 1:1 H₂O:ACN, c(conjugate) = 5 – 25 µM) and subsequently re-crystallized on a polished steel target frame. MALDI-TOF measurements were typically made in reflective positive mode. Spectra were analyzed using Bruker Daltonics flexAnalysis 3.3 (Bruker). Corresponding analytical MS spectra (masses and m/z ratios) are provided in Supporting Table S3 and Supporting Figures S30 and S31.

1.6 Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSA’s) were performed using a Bio-Rad Mini-Protean gel system paired with a direct current (DC) power source (PowerPac™ HC, Bio-Rad). Typically, 6 µL solutions containing RNA (c = 3 µM) and binding partner (c = 4.5 µM) were incubated for 1 h at RT in a binding buffer (1x TAE and 10% glycerol). After incubation, RNA was resolved using 15% non-denaturing polyacrylamide gels (acrylamide:bis-acrylamide (19:1) in 1xTAE) at 150 V in running buffer (1xTAE) at 4 °C for 1.5 hours. For nucleic acid visualization, gels were stained using 2 µL of SYBR™ gold nucleic acid gel dye (Invitrogen) in 20 mL of 1x TAE buffer for 15 min at RT before being visualized using a Bio-Rad ChemiDoc™ (Bio-Rad).

1.7 Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) was conducted using a Malvern MicroCal Auto-iTC200 (Malvern Panalytical). Measurements were performed in 1xPBS and all oligonucleotide, peptide and conjugate samples were dissolved in this buffer beforehand. For experiments with sTAV2b, oligonucleotides (c = 6 µM) were transferred into the sample cell and N-terminally acetylated peptide (c = 108 µM) was
transferred into the syringe. 22 injections per measurement were performed at 25 °C (1.7 μl injection volume, 2 s injection time, 180 s spacing, high feedback mode) with an initial delay of 60 s and a stirring speed of 750 rpm. For experiments involving single stranded DNA, equimolar DNA/peptide solutions or peptide-oligonucleotide conjugates, RNA² (c = 3 – 9 μM) was transferred into the sample cell and corresponding binding partner solutions (c = 30 – 90 μM) were transferred into the syringe. 19 injections per measurement were performed at 25 °C (2 μl injection volume, 2 s injection time, 180 s spacing, high feedback mode) with an initial delay of 60 s and a stirring speed of 750 rpm. Experiments including N-terminally acetylated peptides were performed using the same method where RNA² (c = 9 μM) was transferred into the sample cell and N-terminally acetylated peptide (c = 90 μM) was transferred into the syringe.

Measurements were performed in triplicate. Using the MicroCal LLC ITC software (Origin; OriginLab Corporation), the heat associated with each injection was calculated by integrating the area under the curve in microcalories per second (µcal/s) versus time (min) for each injection and then normalized to concentration. A “single set of identical binding sites” model was used to fit the binding curves from which thermodynamic parameters (ΔG, ΔH, ΔS) and binding parameters (N and K_d) were obtained.

1.8 Circular dichroism spectroscopy & T_m determination

Circular dichroism (CD) spectra were recorded with a Jasco J-1500 spectropolarimeter (Jasco) equipped with a programmable Peltier thermostat in a stoppered quartz cuvette (10 mm; Hellma). Samples were prepared in a buffer of 10 mM sodium phosphate and 100 mM NaCl (pH 7.4). For measurements including sTAV2b, 2:1 mixtures (peptide:oligonucleotide, c(peptide) = 4 μM, c(oligonucleotide) = 2 μM) were prepared. For each sample, 10 CD spectra were measured between 200 nm and 350 nm with continuous scan mode (1 mdeg sensitivity, 1.0 nm resolution, 1.0 nm bandwidth, 2 s integration time, 100 nm/min scan rate). Obtained spectra were averaged and then subtracted from a reference buffer spectrum. CD data were normalized to oligonucleotide strand concentration using Formula 1:

\[ \varepsilon_l - \varepsilon_r = \Delta \varepsilon = \frac{\theta}{32980 \cdot c \cdot l} \]  

where θ is the observed ellipticity in millidegrees, c is the DNA strand concentration in mol·L⁻¹, and l is the path length in cm.

Melting temperature (T_m) determination was conducted using equimolar solutions of given binding partners (c = 2 μM) with the same instrumentation and sample preparation, where ellipticity (θ = 267 nm) was measured by ramping the temperature from 15 – 90 °C (4 °C / min ramp, ±0.05 °C equilibration tolerance, 6 seconds delay after equilibration). Points were taken every 0.5 °C. Raw data were normalized as described above, and T_m-values were determined using the CDpal program⁹ before being plotted in Prism 5.0 (GraphPad).
Melting temperature predictions for RNA/DNA duplexes were performed using the OligoCalc server\textsuperscript{[10]}, applying the same oligonucleotide concentration ($c = 2 \mu M$) and salt concentration ($c = 100 \text{ mM}$) used in experimental measurements. As these predictions do not account for overhanging bases, only the duplex-forming parts of given sequences were included in melting temperature predictions.

1.9 Templated Reactions

For templated reactions, conjugates 3 and 4 as well as RNA templates ($\text{RNA}^{\alpha}$ and $\text{RNA}^{\beta}$) were separately re-dissolved in 100 mM ammonium bicarbonate buffer (reaction buffer; pH 8.0) to provide initial stock solutions ($c = 200 \mu M$). Bis-alkyne crosslinker 5 was dissolved in DMSO ($c = 400 \mu M$). Thereafter, a solution of 8.3 $\mu M$ 3 and 4 in the presence or absence of 8.3 $\mu M$ RNA template ($\text{RNA}^{\alpha}$ or $\text{RNA}^{\beta}$) was prepared in reaction buffer. A separate solution of 5 ($c = 14.4 \mu M$) was prepared in a solution of reaction buffer:acetonitrile solution (33% buffer, 67% acetonitrile). To start the reaction 105 $\mu L$ of conjugate/RNA stock and 70 $\mu L$ of 5 were combined (final concentrations: 5 $\mu M$ 3, 5 $\mu M$ 4-, 5 $\mu M$ RNA, 5.75 $\mu M$ 5; 100 mM ammonium bicarbonate buffer, 20% acetonitrile, 1% DMSO) and incubated at RT. To monitor the reaction, 18 $\mu L$ of reaction solution was added to 82 $\mu L$ of an aqueous 10 mM solution of 3-azido-1-propanamine before being injected on a \textit{RP-HPLC} (1100, \textit{Agilent Technology}. Column:\textit{Agilent AdvancedBio Oligonucleotides}, 4.6 x 150 mm, 2.7 $\mu M$. Solvent A: $\text{H}_2\text{O} + 0.1 \% \text{TFA}$, Solvent B: ACN + 0.1% TFA. Flow Rate: 1 mL·min$^{-1}$, gradient 10 – 50 % B in 10 min) coupled with UV/Vis-detection at $\lambda = 260$ nm. Thereafter, peaks corresponding to reaction products detected at 260 nm ($3, 4, 3*3, 3*4, 4*4, 3* and 4*$) were integrated and the yield was calculated. To verify the identity of ligation product 3$^*4$, the new peak was isolated and analyzed using RP-HPLC (1260 Infinity, \textit{Agilent Technology}. Column: \textit{Agilent InfinityLab Poroshell 120 EC-C18}, 2.1 x 50 mm, 2.7 $\mu M$. Solvent A: $\text{H}_2\text{O} + 0.1 \% \text{TFA}$, Solvent B: ACN + 0.1% TFA. Flow Rate: 0.6 mL·min$^{-1}$) coupled to an ESI-MS (6120 Quadrupole LC/MS, \textit{Agilent Technology}). The same instrumentation was used to analyze crude template reaction mixtures incubated in the absence of RNA. Corresponding chromatograms and analytical mass spectra are provided in Supporting Figures S23 and S24.

To determine the identity of ligation products observed in the absence of RNA, control reactions involving peptides $N_3$-1 and $N_3$-2 were devised. Similar to templated reaction procedure, initial stock solutions of both peptides were prepared in reaction buffer ($c = 200 \mu M$) before being mixed together to yield a 8.3 $\mu M$ intermediate solution. Separately, a solution of bis-alkyne crosslinker 5 ($c = 14.4 \mu M$) was prepared in a solution of reaction buffer:acetonitrile solution (33% buffer, 67% acetonitrile). Peptide and crosslinker solution were mixed accordingly (final concentrations: $N_3$-1 - 5 $\mu M$, $N_3$-2 - 5 $\mu M$, 3 - 5.75 $\mu M$; 100mM ammonium bicarbonate buffer, 20% acetonitrile, 1% DMSO) and incubated at room temperature for 18 hours. Reaction mixtures were analyzed by RP-HPLC (1260 Infinity, \textit{Agilent Technology}. Column: \textit{Agilent InfinityLab Poroshell 120 EC-C18}, 2.1 x 50 mm, 2.7 $\mu M$. Solvent A: $\text{H}_2\text{O} + 0.1 \% \text{TFA}$, Solvent B: ACN + 0.1% TFA. Flow Rate: 0.6 mL·min$^{-1}$) coupled to an ESI-MS (6120 Quadrupole LC/MS, \textit{Agilent Technology}).
Technology). Comparative assignments, chromatograms and analytical mass spectra are provided in Supporting Figures S25.

For time-course measurements, samples of reaction mixtures were analyzed by RP-HPLC at set intervals. Peaks corresponding to reaction products detected at 260 nm (3, 4, 3*3, 3*4, 4*4, 3* and 4*) were integrated and the yield of 3*4 was calculated. Obtained values are given in Supporting Table S6. To calculate initial rates, the yields of three independent experiments were averaged, considering only data points up to 25% yield (and therefore within the linear phase). A linear regression was performed using these points and initial reaction rates in pM·s⁻¹ were determined from the slope (m) of each regression line shown in Supporting Figure S27 according to Formula 2:

$$ v = \frac{c \cdot m}{t} \times 1000 \quad (2) $$

where $t = 1080$ min and $c = 5 \, \mu M$
## 2. Supporting Tables

**Table S1:** Overview of all oligonucleotides with corresponding 5’ and 3’ modifications, sequence (from 5’-end to 3’-end, left to right, 1-letter code) and molecular weight (MW in g/mol). All oligonucleotides bare 5’ or 3’ hydroxyl groups unless otherwise stated. Modifications: P = 5’-terminal phosphate, Thi6 = 6-((6-(λ1-oxidaneyl)hexyl)disulfaneyl)hexan-1-ol linker..

| Oligonucleotide | 5’ Mod. | Sequence (5’ – 3’) | 3’ Mod. | MW (g/mol) |
|-----------------|---------|-------------------|---------|------------|
| RNAα            | P       | UAGCUUAUCAGACUGAUUGA | -       | 7084.2     |
| RNAβ            | P       | CAACAUCCAGUCAUAGCUAU | -       | 7050.2     |
| DNAα            | P       | TAGCTTATCAGACTGATGTTGA | -       | 6764.5     |
| DNAβ            | P       | CAACATCAGTCTGATAAGCTAT | -       | 6782.4     |
| A12             | -       | TCTGATAAGCTA | -       | 3644.4     |
| A12”            |         |                   | Thi6    | 3972.8     |
| A11             | -       | CTGATAAGCTA | -       | 3331.2     |
| A11”            |         |                   | Thi6    | 3668.7     |
| A10             | -       | TGATAAGCTA | -       | 3027.0     |
| A10”            |         |                   | Thi6    | 3379.5     |
| B12             | -       | CAACATCAGTCT | -       | 3589.4     |
| “B12”           | Thi6    |                   |         | 3917.8     |
| B11             | -       | CAACATCAGTC | -       | 3300.2     |
| “B11”           | Thi6    |                   |         | 3628.3     |
| B10             | -       | CAACATCAGT | -       | 2933.2     |
| “B10”           | Thi6    |                   |         | 3315.4     |
**Table S2:** Overview of all synthesized peptides with corresponding N-terminal modification, sequence (from N- to C-terminus), calculated mass-to-charge ratios (m/z calc.) and found masses (m/z found) for charged ions ([M+nH]^{n+}). Amino acids are given in one-letter code. Ac = acetyl, Br = bromoacetamide, N_3 = 6-Azido-hexanoic acid label.

| Peptide   | N-Term. | Sequence (N–C) | m/z calc. | m/z found       |
|-----------|---------|----------------|-----------|-----------------|
| sTAV2b    |         |                 |           |                 |
|           | Ac      | KKQAQRKRHKLNRK| 1048.2    | 1048.1 [M+4H]^4+ |
|           |         | ERGHKSPSEQRRSE|           |                 |
|           |         | LWHAR          |           |                 |
| 1         | Ac      | RKRHKLRHK| 905.7    | 905.6 [M+4H]^4+ |
|           | N_3     | LNRK| 930.0    | 929.8 [M+4H]^4+ |
|           |         | ERGHKSPSEQRRSE|           |                 |
| 1-Br      | Ac      | RKRHKLRHK| 925.7    | 925.2 [M+4H]^4+ |
|           | N_3     | LNRK| 949.9    | 949.7 [M+4H]^4+ |
|           |         | ERGHKSPSEQRRSE|           |                 |
| 2         | Ac      | RKRHKLRHK| 724.7    | 724.6 [M+4H]^4+ |
|           | N_3     | LNRK| 748.9    | 748.9 [M+4H]^4+ |
|           |         | ERGHKSPSEQRRSE|           |                 |
| 2-Br      | Ac      | RKRHKLRHK| 745.6    | 745.4 [M+4H]^4+ |
|           | N_3     | LNRK| 768.4    | 768.7 [M+4H]^4+ |
|           |         | ERGHKSPSEQRRSE|           |                 |
**Table S3**: Overview of synthesized peptide-oligonucleotide conjugates with corresponding N-terminal peptide modification, peptide sequence (from N- to C-terminus), oligonucleotide sequence (from 5’ to 3’) calculated mass-to-charge ratios (m/z calc.) and found masses (m/z found) for charged ions ([M+nH]n+). Amino acids are given in one-letter code. Ac = acetyl, N3 = 6-Azido-hexanoic acid label, Thi6 = 6-(6-(λ1-oxidaneyl)hexyl)disulfaneyl)hexan-1-ol linker, ^ = peptide and oligonucleotide positions connected through a thioether linkage, *= peptide positions connected through a DBCO-PEG4-DBCO linker.

| POC | N-Term. | Peptide Sequence (N – C) | Oligonucleotide Sequence (5’ – 3’) | m/z calc. | m/z found |
|-----|---------|-------------------------|-----------------------------------|-----------|-----------|
| 1-A12 | Ac      | RKRHKLRKGERGHKSPSEQRRSELWHAK^ | TCTGATAAGCTA(Thi6)^ | 829.4 | 829.7 [M+9H]9+ |
|      | N3      |                         |                                   | 1511.5 | 1512.1 [M+5H]5+ |
| 1-A11 | Ac      | RKRHKLRKGERGHKSPSEQRRSELWHAK^ | CTGATAAGCTA(Thi6)^ | 1431.3 | 1431.8 [M+5H]5+ |
| 1-A10 | Ac      | RKRHKLRKGERGHKSPSEQRRSELWHAK^ | TGATAAGCTA(Thi6)^ | 1373.5 | 1374.0 [M+5H]5+ |
| 2-B12 | Ac      | RKRHKLRKGERGHKSPSEQRRK^ | ^ (Thi6)CAACATCAGTCT | 742.9 | 742.5 [M+9H]9+ |
|      | N3      |                         |                                   | 1355.9 | 1356.0 [M+5H]5+ |
| 2-B11 | Ac      | RKRHKLRKGERGHKSPSEQRRK^ | ^ (Thi6)CAACATCAGTC | 3187.3 | 3186.3 [M+2H]2+ |
| 2-B10 | Ac      | RKRHKLRKGERGHKSPSEQRRK^ | ^ (Thi6)CAACATCAGT | 6084.3 | 6085.2 [M+H]1+ |
| 3*4  |         | *RKRHKLRKGERGHKSPSEQRRSELWHAK^ | TCTGATAAGCTA(Thi6)^ | 894.1 | 894.2 [M+17H]17+ |
|      |         | *RKRHKLRKGERGHKSPSEQRRK^ | ^ (Thi6)CAACATCAGTCT |     |           |
Table S4: Overview of isothermal titration calorimetry data. Equilibrium dissociation constant ($K_d$), binding stoichiometry ($N$), enthalpy of binding ($\Delta H$), entropy of binding ($-T\Delta S$), and Gibbs free energy of binding ($\Delta G$) are presented. Measurements were performed in triplicate. * denotes binding partner (BP’s) co-incubated in the cell or syringe.

| BP 1    | BP 2    | BP 3    | $K_d$ / µM | $N$     | $\Delta H$ / kcal·mol$^{-1}$ | $-T\cdot\Delta S$ / kcal·mol$^{-1}$ | $\Delta G$ / kcal·mol$^{-1}$ |
|---------|---------|---------|------------|---------|-------------------------------|-------------------------------------|-------------------------------|
| RNA$^a$ | RNA$^b$ | sTAV2b  | 0.089 ±0.01| 2.12    | -30.3 ±0.90                    | 20.7 ±0.96                         | -9.6 ±0.07                    |
| RNA$^a$ | DNA$^b$ | sTAV2b  | 0.599 ±0.03| 2.34    | -23.2 ±0.90                    | 20.9 ±0.83                         | -2.2 ±1.23                    |
| RNA$^a$ | A12     | -       | 0.319 ±0.05| 1.13    | -34.4 ±0.76                    | 25.5 ±0.86                         | -8.8 ±0.10                    |
| RNA$^a$ | A12     | Ac-1    | 0.420 ±0.08| 0.9     | -30.0 ±0.92                    | 21.3 ±1.01                         | -8.7 ±0.10                    |
| RNA$^a$ | 1-A12   | -       | 0.004 ±0.002| 0.86    | -78.2 ±0.69                    | 66.8 ±0.81                         | -11.5 ±0.40                   |
| RNA$^a$ | B12     | -       | 0.074 ±0.001| 1.02    | -36.9 ±0.61                    | 27.1 ±0.58                         | -9.7 ±0.10                    |
| RNA$^a$ | B12     | Ac-2    | 0.087 ±0.004| 0.98    | -34.3 ±0.67                    | 24.6 ±0.71                         | -9.6 ±0.04                    |
| RNA$^a$ | 2-B12   | -       | 0.004 ±0.001| 0.79    | -40.9 ±1.25                    | 29.3 ±1.06                         | -11.7 ±0.20                   |
Table S5: Overview of predicted melting temperatures ($T_m$) for DNA/RNA duplexes, observed melting temperatures ($T_{m}^{*}$) for DNA/RNA duplex sequences and Conjugate/RNA duplexes. A15 and B18 correspond to predicted complementary sequences with closest melting temperatures to those of 1-A12 and 2-B12 respectively. | denotes the cut-off point for overhanging bases included in the melting temperature prediction.

| Duplex | Sequences (5’ – 3’)/(3’ – 5’) | $T_{m}^{*}$ / °C | $T_m$ / °C | $T_{m}^{\text{Conj}}$ / °C |
|--------|-------------------------------|----------------|----------|-----------------|
| RNA\textsuperscript{a} A12 | UAG CUU AUC AGA | 37 | 36 | 47 |
|         | ATC GAA TAG TCT               |               |          |                 |
| RNA\textsuperscript{a} A11 | UAG CUU AUC AG | 32 | 35 | 47 |
|         | ATC GAA TAG TC               |               |          |                 |
| RNA\textsuperscript{a} A10 | UAG CUU AUC A | 25 | <32 | 40 |
|         | ATC GAA TAG T               |               |          |                 |
| RNA\textsuperscript{a} A15 | UAG CUU AUC AGA CUG | 48 | - | - |
|         | ATC GAA TAG TCT GAC         |               |          |                 |
| RNA\textsuperscript{a} B12 | AGA CUG AUG UUG | 39 | 44 | 53 |
|         | TCT GAC TAC AAC             |               |          |                 |
| RNA\textsuperscript{a} B11 | GA CUG AUG UUG | 34 | 44 | 49 |
|         | CT GAC TAC AAC             |               |          |                 |
| RNA\textsuperscript{a} B10 | GA CUG AUG UUG | 28 | 36 | 43 |
|         | CT GAC TAC AAC             |               |          |                 |
| RNA\textsuperscript{a} B18 | CUU AUC AGA CUG AUG UUG | 54 | - | - |
|         | GAA TAG TCT GAC TAC AAC     |               |          |                 |
Table S6: Time-dependent yields of ligation product 3*4 based on three independent experiments (Expt.) where 3, 4 and 5 were incubated in the presence of RNA\(^\alpha\) (+RNA\(^\alpha\)), in the absence of RNA (No RNA\(^\alpha\)), and in the presence of RNA\(^\beta\) (+RNA\(^\beta\)).

| Time / min | + RNA\(^\alpha\) Yield (3*4) / % | No RNA Yield (3*4) / % | + RNA\(^\beta\) Yield (3*4) / % |
|------------|---------------------------------|------------------------|---------------------------------|
|            | Expt 1. | Expt 2. | Expt 3 | Expt 1. | Expt 2. | Expt 3 | Expt 1. | Expt 2. | Expt 3 |
| 10         | 4       | 1       | 2      | -       | -       | -      | -       | -       | -      |
| 15         | 14      | 11      | 10     | -       | -       | -      | -       | -       | -      |
| 20         | 25      | 23      | 22     | -       | -       | -      | -       | -       | -      |
| 40         | 33      | 28      | 26     | -       | -       | -      | -       | -       | -      |
| 60         | 38      | 32      | 31     | -       | -       | -      | -       | -       | -      |
| 360        | 59      | 59      | 67     | -       | -       | -      | -       | -       | -      |
| 1080       | 96      | 94      | 98     | -       | -       | -      | -       | -       | -      |
| 10         | -       | -       | -      | -       | -       | -      | -       | -       | -      |
| 15         | -       | -       | -      | -       | -       | -      | -       | -       | -      |
| 20         | 1       | 3       | 2      | -       | -       | -      | -       | -       | -      |
| 40         | 2       | 4       | 3      | -       | -       | -      | -       | -       | -      |
| 60         | 4       | 5       | 6      | -       | -       | -      | -       | -       | -      |
| 360        | 12      | 9       | 10     | -       | -       | -      | -       | -       | -      |
| 1080       | 29      | 29      | 27     | -       | -       | -      | -       | -       | -      |
| 10         | -       | -       | -      | -       | -       | -      | -       | -       | -      |
| 15         | -       | -       | -      | -       | -       | -      | -       | -       | -      |
| 20         | 2       | 3       | 4      | -       | -       | -      | -       | -       | -      |
| 40         | 3       | 5       | 4      | -       | -       | -      | -       | -       | -      |
| 60         | 4       | 7       | 5      | -       | -       | -      | -       | -       | -      |
| 360        | 17      | 9       | 10     | -       | -       | -      | -       | -       | -      |
| 1080       | 29      | 33      | 31     | -       | -       | -      | -       | -       | -      |
3. Supporting Figures

**Figure S1**: ITC measurements of N-terminally acetylated sTAV2b with double-stranded RNAα/RNAβ.

Measurements were performed in triplicate (c(peptide) = 108 μM and c(RNAα/RNAβ) = 6 μM).

RNAα/RNAβ + sTAV2b measurement 1

RNAα/RNAβ + sTAV2b measurement 2

RNAα/RNAβ + sTAV2b measurement 3
Figure S2: ITC measurement of N-terminally acetylated sTAV2b with double-stranded DNA$^\alpha$/DNA$^\beta$, (c(peptide) = 108 μM and c(DNA$^\alpha$/DNA$^\beta$ = 108 μM).
Figure S3: ITC measurements of N-terminally acetylated sTAV2b with double-stranded RNAα/DNAβ. Measurements were performed in triplicate (c(peptide) = 108 μM and c(RNAα/DNAβ) = 6 μM).
**Figure S4:** CD spectra of double-stranded RNA\(^\alpha/\alpha\)\(^\beta\), RNA\(^\alpha/DNA\(^\beta\) and DNA\(^\alpha/DNA\(^\beta\).

**Figure S5:** CD spectra of N-terminally acetylated peptide sTAV2b, spectra of double-stranded RNA\(^\alpha/\alpha\)\(^\beta\), spectra of double-stranded RNA\(^\alpha/\alpha\)\(^\beta\) with sTAV2b in solution (RNA\(^\alpha/\alpha\)\(^\beta\)/sTAV2b) and the sum of the two spectra (RNA\(^\alpha/\alpha\)\(^\beta\) + sTAV2b).
**Figure S6**: Polar contact matrix corresponding to arrangement A based on interactions observed in the crystal structure of TAV2b in complex with a palindromic RNA duplex (PDB ID: 2ZI0). Colored tiles represent hypothetical polar contacts between amino acids of the sTAV2b sequence with nucleotides of either duplex strand - RNA° and DNA® (gray and blue respectively). Highlighted in boxes are the contacts associated with a 28-mer peptide and a duplex containing 12-mer DNA strand.
Figure S7: Polar contact matrix corresponding to arrangement B based on interactions observed in the crystal structure of TAV2b in complex with a palindromic RNA duplex (PDB ID: 2ZI0). Colored tiles represent hypothetical polar contacts between amino acids of the sTAV2b sequence with nucleotides of either duplex strand - RNA^α and DNA^β (gray and blue respectively). Highlighted in boxes are the contacts associated with a 21-mer peptide and a duplex containing 12-mer DNA strand.
Figure S8: EMSA of A12, an equimolar solution of A12 and peptide 1 (A12 + 1) and 1-A12 (c = 4.5 μM, for each ligand) incubated in the presence of absence of RNAα (c = 3 μM). Gel imaged after SYBR™ gold staining. Seen at the bottom of lanes 3-6 is unbound, single-stranded A12.
Figure S9: ITC measurements of A12 with RNAα. Measurements were performed in triplicate (c(A12) = 90 μM and c(RNAα) = 9 μM).
Figure S10: ITC measurements of an equimolar A12 + 1 solution with RNAα. Measurements were performed in triplicate (c(A12+1) = 90 μM and c(RNAα) = 9 μM).
Figure S11: ITC measurements of a 1-A12 with RNA\textsuperscript{0}. Measurements were performed in triplicate (c(1-A12) = 30 μM and c(RNA\textsuperscript{0}) = 3 μM).
Figure S12: EMSA of B12, an equimolar solution of B12 and peptide 2 (B12 + 2) and 2-B12 (c = 4.5 μM, for each ligand) incubated in the presence of absence of RNAα (c = 3 μM). Gel imaged after SYBR™ gold staining. Unbound, single-stranded B12 migrated out of the gel and was therefore not visible in lanes 3 – 6.
Figure S13: ITC measurements of a B12 with RNA°. Measurements were performed in triplicate ($c$(B12) = 90 $\mu$M and $c$(RNA°) = 9 $\mu$M).
Figure S14: ITC measurements of an equimolar B12 + 2 solution with RNA°. Measurements were performed in triplicate (c(B12+2) = 90 μM and c(RNA°) = 9 μM).
Figure S15: ITC measurements of an equimolar 2-B12 solution with RNA\(^{a}\). Measurements were performed in triplicate (c(2-B12) = 30 μM and c(RNA\(^{a}\)) = 3 μM).
Figure S16: Synthetic route to the bromoacetamide-modified analogs of peptides 1 and 2 (1-Br and 2-Br, respectively). Peptides were synthesized according to standard Fmoc-based SPPS protocols.
Figure S17: (a) Thiol-modified analogs of DNA strands A12 and B12 (A12'' and B12 respectively). (b) Peptide-DNA conjugation protocol for the synthesis of 1-A12.
Figure S18: Melting temperature profiles of RNA$^\alpha$ in the presence of A and B series DNA truncates, equimolar mixtures of DNA and peptide (either 1 or 2) and conjugate truncates respectively ($\lambda = 267$ nm, $c$(RNA$^\alpha$) = 2 $\mu$M, $c$(ligand) = 2 $\mu$M, buffer: 10 mM sodium phosphate, pH 7.4, 100 mM NaCl). (a) A11 measurements, (b) B11 measurements, (c) A10 measurements, (d) B10 measurements.
Figure S19: EMSA of RNAα (c = 3 μM) in the presence of different conjugates including co-incubation with A- and B-series members (c = 4.5 μM, for each ligand) baring DNA sequences of equal lengths (X). Please note, the three lanes on the far right of the gel correspond to RNAα (c = 3 μM) in the presence of peptides 1 and 2 as well as DNA sequences of equal length (X) from both series A and B (c = 4.5 μM, for each ligand).
Figure S20: Diagrams of conjugate pairs binding RNA\(^\alpha\) highlighting terminal base pairs (black dashed lines). (a) RNA\(^\alpha\) bound to 1-A12 and 2-B12. (b) RNA\(^\alpha\) bound to 1-A11 and 2-B11. (c) RNA\(^\alpha\) bound to 1-A10 and 2-B10.
Figure S21: Chemical structure of the bis-alkyne crosslinker DBCO-PEG4-DBCO, referred to as 5.

Figure S22: (a) Diagram of 3 highlighting N-terminal azide modification (b) Diagram of 4 highlighting N-terminal azide modification.
Figure S23: Structure of ligation product 3*4 including the chemical structure of the reacted crosslink and corresponding ESI-MS assignment including calculated and found m/z ratios measured from purified sample.
Figure S24: Structure of ligation product 3*3 including the chemical structure of the reacted crosslink and corresponding ESI-MS assignment including calculated and found m/z ratios measured from crude reaction mixture after 18 h.
Figure S25: (a) Schematic overview of control ligation reaction. Azide-modified analogues of 1 and 2 (N₃-1 and N₃-2 respectively) react with the bis-alkyne crosslinker 5 to form a mixture of ligation products including homodimers 1₁¹ and 2₂², heterodimer 1₁² and singly-reacted peptides 1* and 2*. (b) (Upper) HPLC trace of reaction between 1*, 2* and 5 after 18 h in the absence of RNA highlighting peaks that could not be assigned by ESI-MS. (Lower) HPLC trace of control ligation between N₃-1 and N₃-2 and 3 after 18h. (c) ESI-MS assignment of 2*² including calculated and found m/z ratios. (d) ESI-MS assignment of 1* and 2* including calculated and found m/z ratios.
Figure S26: EMSA of A12, an equimolar solution of A12 and peptide 1 (A12 + 1), 1-A12, B12, an equimolar solution of B12 and peptide 2 (B12 + 2), 2-B12 (c = 4.5 μM, for all ligands) incubated in the presence of absence of RNAβ (c = 3 μM). Gel imaged after SYBR™ gold staining.
Figure S27: Linear fit of data obtained from three independent, time-course experiments. Initial rates ($V_i$) are based on slope of these fits using formula 2. (a) RNA$^\alpha$, $v_r = 100 \pm 7$ pM·s$^{-1}$ (b) No RNA, $v_r = 1.0 \pm 0.1$ pM·s$^{-1}$ (c) RNA$^\beta$, $v_r = 1.1 \pm 0.2$ pM·s$^{-1}$.
Figure S28: HPLC chromatograms ($\lambda = 210$ nm) including peak retention time and corresponding mass spectra of N-terminally acetylated peptides (sTAV2b, Ac-1 and Ac-2) used for EMSA experiments, ITC measurements and melting temperature experiments.
Figure S29: HPLC chromatograms ($\lambda = 210$ nm) including peak retention time and corresponding mass spectra of peptides of $N$-terminally acetylated and $N$-terminally azide-labelled peptides equipped with side chain bromoacetamide handles (Ac-1-Br, N3-1-Br, Ac-2-Br and N3-2-Br) used for peptide-oligonucleotide conjugation reactions.
Figure S30: HPLC chromatograms ($\lambda = 260$ nm) including peak retention time and corresponding mass spectra of N-terminally acetylated peptide-oligonucleotide conjugates (1-A12, 1-A11 and 1-A10) used for EMSA experiments, ITC measurements and melting temperature experiments.
Figure S31: HPLC chromatograms ($\lambda = 260$ nm) including peak retention time and corresponding mass spectra of $N$-terminally acetylated peptide-oligonucleotide conjugates (2-B12, 2-B11 and 2-B10) used for EMSA experiments, ITC measurements and melting temperature experiments.
Figure S32: HPLC chromatograms ($\lambda = 260$ nm) including peak retention time and corresponding mass spectra of N-terminally azide-labelled peptide-oligonucleotide conjugates (3 and 4) used for templated reactions.

Figure S33: HPLC chromatograms ($\lambda = 210$ nm) including peak retention time and corresponding mass spectra of N-terminally azide-labelled peptides (N$_3$-1 and N$_3$-2) used for control reactions.
4. Supporting References

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