Discovery of G-quadruplex stabilizing ligands through direct ELISA of a one-bead-one-compound library†

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We describe the identification of small-molecule G-quadruplex ligands using a direct ELISA screen of a one-bead-one-compound library of unnatural polyamides displayed on a branched linker with a biotin tag. This general purpose parallel screen for small molecule–oligonucleotide interactions was validated by surface plasmon resonance and ELISA of resynthesized compounds. Linear polyamides displayed similar rankings in their affinity for quadruplex as their branched counterparts. Quadruplex affinity as judged by these surface based techniques was a useful predictor of the ability of the ligands to stabilize the quadruplex to thermal unfolding in solution.

Introduction

Guanine rich sequences with the potential to adopt a quadruplex fold have been located computationally at numerous positions in the human genome,[1] and experimentally confirmed for sequences from the telomere[2] and within the promoters of certain proto-oncogenes.[3,4] Telomeric DNA attrition on cell replication ultimately leads to cell senescence, although in the majority of cancers telomeres are maintained by the enzyme telomerase, which is expressed in greatly reduced levels in somatic cells.[5] The action of telomerase is inhibited by the folding of its single stranded DNA substrate into a quadruplex,[6] which may also displace proteins which protect the telomere from being falsely recognized as damaged DNA.[7] These observations, coupled with evidence that quadruplex formation in the promoter of the *c-myc* proto-oncogene may lead to down-regulation of gene expression,[4] and the possibility of similar effects for other genes containing potential quadruplex sequences in their DNA or mRNA have stimulated interest in small molecules that bind to, and stabilize, quadruplexes. Such compounds have the potential for arrest of cancer cell growth through disruption of telomere maintenance and alteration of oncogene expression levels.

As a means for discovery of quadruplex ligands, the one-bead-one-compound solid-phase library synthesis approach conveniently affords individual compounds for miniaturized screening. Here we demonstrate a new approach to identification of small-molecule oligonucleotide ligands from one-bead-one-compound libraries with a generally applicable enzyme linked immunosorbent assay (ELISA) against a surface immobilized telomeric quadruplex and confirm the ability of the compounds to stabilize the quadruplex from thermal unfolding in solution.

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ELISA offers a rapid and readily automated means of assaying binding of ligands to surface immobilized targets, in this instance a folded oligonucleotide. Rather than screening a bead-supported library[8] we opted to screen solution phase ligands, cleaved from individual library beads, against surface immobilized DNA. This approach provides stocks of ligands that can be assayed in parallel against multiple targets, and subjected to tandem mass spectrometry to determine compound identity. As a screening method against a new target, competitive ELISA suffers from the disadvantage of requiring both a known ligand and an associated antibody. In addition, the ligand–target interaction must be inhibited by the library compounds[9] which necessitates a common binding site, or allosteric communication between sites. Due to these limitations we have instead chosen to develop a direct ELISA for detection of target-bound ligands by appending to the ligand a tagging group that is recognized by an enzyme conjugate. Polyamides are an attractive class of ligand molecule due to their ease and rapidity of construction using solid phase protocols and the multitude of amino acid building blocks available for introduction of diversity during library construction. The direct ELISA assay was exploited for screening of polyamide ligands from a split-and-pool one-bead-one-compound synthesis against a DNA target consisting of five GTTAGG repeats of the human telomeric sequence, folded into a quadruplex (Htelo). The outcome of the screen was validated using surface plasmon resonance binding studies and an established assay based on thermal melting of a fluorophore labelled quadruplex in solution.

Results and discussion

A scaffold was designed that would enable combinatorial construction of quadruplex binding polyamide moieties and permit detection of oligonucleotide binding using an ELISA. In order to enhance binding affinity, a bivalent display of peptides on a branched linker was adopted. The bifurcation was provided by a lysine side chain[10] through which the library peptides were attached *via* flexible tri-glycine spacers (Fig. 1). For the purpose of detection, a single tag group was introduced at the C-terminus prior to the branch point. The FLAG peptide[11] (DYKDDDDK) and Glu(PEG-biotin) tags were evaluated as these are conveniently

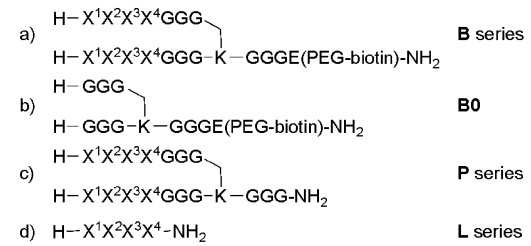

Fig. 1 General structures of branched and linear peptides.

introduced in an initial step during solid phase synthesis and both tags are robust to subsequent synthetic manipulations. The Glu(PEG-biotin) tag combined with detection using a streptavidin–horseradish peroxidase polymer conjugate proved preferential, as in trials FLAG tagged compounds generated comparatively lower signals (data not shown) which are likely to arise from reduced binding due to repulsion between the highly negatively charged FLAG sequence and the polyanionic DNA target. Detection using streptavidin–horseradish peroxidase polymer was found to offer superior sensitivity to antibody mediated detection with a horseradish peroxidase anti-biotin conjugate.

On the basis of the observation of quadruplex complexes with the natural product distamycin,[12] acridines,[13] and our own work on quadruplex binding peptides,[14] we selected a number of natural and unnatural amino acids with which to construct a biased library of polyamide ligands with a predisposition towards quadruplex binding. Aromatic and positively charged groups are a typical feature of oligonucleotide ligands, forming π-stacking interactions, hydrophobic and hydrogen bonding groove contacts and electrostatic interactions with the phosphate backbone. For the purpose of our library, aromatic building blocks included tyrosine, tryptophan, histidine, methylimidazole, methylpyrrole[15] and an acridine amino acid.[16] Arginine and lysine provided positive charge, and valine was included to allow hydrophobic contacts with exposed bases and sugars. The library was prepared on a macrobead resin with the biotin tagged branched scaffold (Fig. 1a) using the split-and-pool method where X^1 = Tyr, Trp, Phe, Acr, X^2 = Arg, Im, Lys, X^3 = His, Lys, Py and X^4 = Arg, Val, Tyr.[17] The structures of the non-proteinogenic amino acids are given in Fig. 2. A random selection of crude peptides cleaved from the solid support was screened in parallel at a single concentration in potassium phosphate buffer (pH 7.4, 190 mM K^+) by ELISA against the Htelo quadruplex (GTT AGG)$_5$, and a non-quadruplex sequence of identical length (AGT TAG)$_5$, as a polyanionic background. The ELISA response, as measured by the absorbance at 450 nm, is expected to increase with the affinity of the peptide for the nucleic acid, thereby enabling library members to be ranked accordingly. The ratio of ELISA absorbance measurements between the quadruplex and non-quadruplex oligonucleotide was taken as an indicator of the specificity of the peptide for the quadruplex. On the basis of these measurements a selection of compounds spanning the range of affinity to the Htelo quadruplex were subjected to MALDI-TOF/TOF mass spectrometric analysis to determine their sequences. The majority of sequences that displayed selectivity for Htelo contained a single positively charged residue, whereas compounds that bound comparably to the background possessed multiple charged residues, indicating that excessive electrostatic interactions can lead to a reduced specificity.

Eight compounds (**B1–B8**) identified in the initial screen and spanning the range of apparent Htelo affinity were chosen for validation. ELISA titrations were performed against Htelo using resynthesized and HPLC purified peptides to derive apparent association constants (Table 1). Peptide **B0** (Fig. 1b), which consists of only the tag and branched linker, was used as a blocking agent during these titrations so as to block sites that bind these components of the ligands. For comparison of quadruplex *versus* duplex affinity, ELISA titrations against a 22 base pair DNA duplex (Dup) were performed but resulted in relatively reduced signals (<50% compared to Htelo), with the exception of **B8**, which can be attributed to weaker interactions and/or fast dissociation during wash steps. The rank order of compound affinity according to the quantitative ELISA is in broad agreement with that inferred from the initial screen, thereby confirming that these measurements with crude compounds are representative and that the compounds have been correctly identified.

Surface plasmon resonance (SPR) provides an alternative means of monitoring the interaction of a solution phase ligand with an immobilized oligonucleotide.[18] Compounds **P1–P8** were prepared as analogues of **B1–B8** for SPR analysis. These compounds lack a biotin tag which is both unnecessary for SPR and may lead to complications due to detection of direct binding of the tag to the streptavidin coated chip. The equilibrium binding interactions of compounds **P1–P8** were quantified by SPR against Htelo and Dup under the same buffer conditions used for ELISA. Increasing the concentration of peptide to 100 μM did not achieve binding saturation, although high responses indicated either multiple binding sites per DNA or compound aggregation. At compound concentrations <10 μM the equilibrium response was approximately linear, allowing an estimate of the affinity (Table 1) to be determined from the gradient of a linear fit.

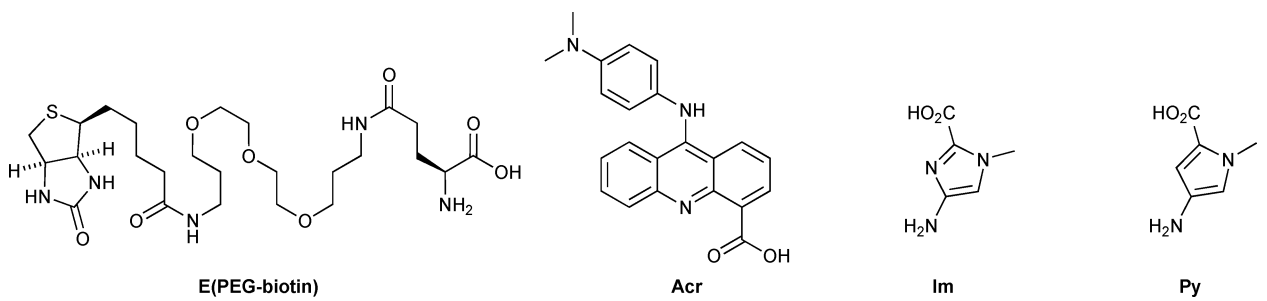

Fig. 2 Amino acid structures.

Table 1 Sequences of selected compounds and their affinities for Htelo by ELISA compared with SPR (Htelo and duplex) and FRET melting at 4 μM

Sequence[a] (X^1 X^2 X^3 X^4)	A450[b]	ELISA[c] log(K_a)	SPR log(K_a) **P** series		ΔT_m/°C[e]
			Htelo	Dup	
1 YRHY	0.23	5.7	4.7	[d]	1.9
2 WR–Py–Y	0.25	6.3	5.3	[d]	6.0
3 FRKV	0.34	6.8	4.4	[d]	4.8
4 Y–Im–Py–R	0.21	6.6	5.4	4.8	15.1
5 Y–Im–KV	0.07	5.2	4.3	[d]	1.4
6 Y–Im–HR	0.27	6.3	4.8	[d]	3.4
7 WK–Py–Y	0.27	6.4	5.0	[d]	4.3
8 WKKR	0.43	7.4	5.7	4.5	21.6

[a] Py is 1-methylpyrrole, and Im is 1-methylimidazole. [b] Absorbance at 450 nm in a single point ELISA library screen of **B** series against Htelo. [c] **B** series, against Htelo. [d] Absence of detectable binding above background. Log(K_a) <3.7 based on the instrument detection limit. [e] Shift in T_m of dual labelled telomeric quadruplex in cacodylate buffer (pH 7.4, 50 mM KCl), **P** series.

In comparison with the SPR data, the ELISA clearly enables identification of the sequences with the highest (**8**), lowest (**5**) and intermediate affinities for the Htelo quadruplex. The apparent affinities for Htelo determined by SPR are lower than those obtained in the corresponding ELISA. This reflects the ability of SPR to observe all binding events simultaneously *in situ*, whereas ELISA is biased towards detection of interactions with sufficiently low off-rates to survive dissociation during wash steps. SPR confirmed our supposition that there is significant discrimination against the duplex. This is consistent with a mode of binding involving stacking and hydrophobic contacts with bases which are inaccessible in the duplex but are exposed at the terminal tetrads and loops of a quadruplex. Differences are observed between the affinities of peptides with an identical number of positive charges at pH 7.4, for example **P4** and **P5**, suggesting that binding is not solely driven by electrostatic interactions with the phosphate backbone.

The impact of the branched linker was tested using linear tetrapeptides (**L1–L8**) in which this linker was absent. SPR data were analyzed analogously to the branched polyamides (Table 2). For Htelo, there is a strong correspondence between log(K_a) for the two series of peptides, suggesting similar interaction modes. The K_a for the branched series are less than that expected for simultaneous independent binding of two corresponding linear peptides and the selectivity of the linear peptides is reduced, with these compounds demonstrating relatively enhanced affinity for the duplex. These results suggest that the linker disfavours binding to both quadruplex and duplex, with a more pronounced effect for duplex, thus enhancing the overall quadruplex selectivity. The structure of **L4** consists of two linked heterocyclic residues and a C-terminal arginine, which is reminiscent of known minor groove binders such as distamycin, suggesting that the affinity of this compound for duplex may also arise from interactions with the minor groove.

Table 2 Affinities of linear peptides for Htelo and Dup determined by SPR

	Sequence	SPR log(K_a) **L** series	
		Htelo	Dup
L1	YRHY	3.6	3.1
L2	WR–Py–Y	4.2	3.6
L3	FRKV	3.3	3.2
L4	Y–Im–Py–R	4.6	4.3
L5	Y–Im–KV	3.4	2.8
L6	Y–Im–HR	3.8	3.3
L7	WK–Py–Y	4.0	3.4
L8	WKKR	4.4	3.9

The ability of the branched polyamides **P1–P8** to stabilize the telomeric quadruplex to thermal unfolding in solution was investigated with an established FRET assay using a dual labelled oligonucleotide.[19] On melting the average distance between the fluorophores increases leading to a decrease in energy transfer efficiency. The melting temperature was found to increase as a function of polyamide concentration, indicating the ability of the polyamide to stabilize a folded conformation of the DNA. The shifts in melting temperature (ΔT_m) at 4 μM peptide are given in Table 1. It is informative to compare these data with the ELISA and SPR to validate the ability of these surface based affinity techniques to predict the relative abilities of ligands to stabilize the quadruplex in free solution. There is a broad consensus between the rankings of the sequences, in agreement with the expectation that quadruplex stabilization will increase with ligand affinity. We have quantified the extent to which melting temperature correlates with SPR and ELISA by calculation of Kendall's rank correlation coefficient, τ, which varies between -1 for perfect rank reversal between two data sets, and 1 for perfect rank agreement. τ of ΔT_m against log(K_a) is 0.71 and 0.79 for ELISA and SPR respectively, both values being significant at the 5% level in a two sided test. This indicates that the techniques are in useful agreement for the purposes of ranking the relative affinity of ligands in screens.

Conclusions

Through the use of a bifurcated linker with tri-glycine spacers and a biotin tag, it was possible by a single point ELISA to rank tetrapeptide ligands according to binding to a DNA quadruplex. The off-rate of ligands displayed on this scaffold is sufficiently low that a signal is still obtained from the ELISA despite a number of wash steps during which dissociation could occur. The initial single-point ELISA screen of a library was performed with crude material, cleaved directly from macrobeads, and may therefore contain remnants of protecting groups. However, when the assay was repeated at multiple-concentrations using a selection of resynthesized and purified compounds that were identified in

the original screen, a broadly similar ranking of the quadruplex affinity was measured. These results demonstrate that the single-point ELISA of crude material is representative of the data obtained from pure compounds hence confirming the value of the ELISA as a screen. The ELISA is particularly suited for parallel screening against multiple oligonucleotides thus enabling ligands which also bound to a non-quadruplex DNA to be identified simultaneously.

Surface based techniques, such as ELISA, which involve a solid substrate and a label for detection can be susceptible to interference arising from undesirable interactions between the substrate and label with the ligand or target. Rankings of compounds lacking a biotin tag by surface plasmon resonance and a solution FRET melting assay were in good agreement with each other, demonstrating minimal interference arising from immobilization of the oligonucleotide to a surface. Correlation between FRET and ELISA of biotinylated ligands was less strong, but nevertheless useful. The SPR results with simple tetrapeptides demonstrate that while the branching construct does enhance quadruplex binding, it does so to a lesser extent than might be predicted, and also appears to enhance discrimination against duplex. The former may arise due to the site separation of immobilized oligonucleotides preventing both peptide moieties from simultaneously adopting optimum binding contacts. The latter effect may stem from the linker sterically inhibiting insertion of heterocyclic residues into the minor groove of the duplex. Based on a count of positively charged residues in the compounds and the observed variability in binding affinity towards quadruplex, the mode of quadruplex binding cannot be solely attributed to electrostatic interactions with the DNA backbone, indicating that aromatic stacking and hydrophobic interactions play a role.

Experimental

General

Fmoc protected amino acids were supplied by Novabiochem, except for methylimidazole and methylpyrrole residues which were prepared according to the literature.[15] $POCl_3$ was distilled prior to use. Triethylamine was distilled *ex* CaH_2. *N*-Dimethylphenylenediamine was distilled using a Kugelrohr apparatus. Mass spectrometry was performed on an Applied Biosystems 4700 Proteomics Analyzer (MALDI-TOF/TOF) and Micromass Q-Tof spectrometers. ^1H and ^{13}C NMR spectra were recorded on Bruker DRX400 and Avance 500 Cryo spectrometers. Coupling constants are given in Hertz.

Oligonucleotides. Htelo 5′ Biotin-GTT AGG GTT AGG GTT AGG GTT AGG GTT AGG 3′. Bck 5′ Biotin-AGT TAG AGT TAG AGT TAG AGT TAG AGT TAG 3′. Dup 5′ Biotin-GGC ATA GTG CGT GGG CGT TAG C 3′ hybridized to 5′ GCT AAC GCC CAC GCA CTA TGC C 3′. F21T 5′ FAM-GGG TTA GGG TTA GGG TTA GGG-TAMRA 3′

Desalted biotinylated oligonucleotides were supplied by Invitrogen. Stock solutions were standardized using the absorbance at 260 nm. For SPR, biotinylated Htelo at a concentration of 10 μM was annealed in buffer (150 mM KCl, 50 mM TrisHCl, pH 7.4) by cooling from 95 °C to room temperature over 3 h, followed by incubation at 4 °C overnight. Annealing for ELISA was performed similarly using oligonucleotide at a nominal concentration of 20 μM in phosphate buffer (100 mM potassium phosphate, 50 mM KCl, pH 7.4). Annealed stocks were standardized using the absorbance at 260 nm.

Synthesis

The acridine amino acid was prepared from 9-oxo-9,10-dihydro-acridine-4-carboxylic acid isopropyl ester using a modification of a literature procedure for a closely related compound.[16]

9-(4-Dimethylamino-phenylamino)-acridine-4-carboxylic acid isopropyl ester. $POCl_3$ (0.54 cm^3) and triethylamine (3.2 cm^3) were added dropwise over 5 min to a stirred solution of 1,2,4-triazole (1.31 g) in acetonitrile at 0 °C under Ar. The resulting suspension was allowed to warm to rt over 30 min and a solution of 9-oxo-9,10-dihydro-acridine-4-carboxylic acid isopropyl ester (584 mg, 2.1 mmol) in dichloromethane (4 cm^3) added. The solution was refluxed under Ar overnight, allowed to cool slightly, then water (2.8 cm^3) and triethylamine (8.5 cm^3) carefully added down the condenser. After refluxing for a further 10 min, the solution was evaporated under reduced pressure to an orange paste. This was dissolved in dichloromethane (50 cm^3), filtered, washed with sodium bicarbonate (satd. aq, 50 cm^3), and brine (50 cm^3). The organic phase was dried (Na_2SO_4), evaporated, and the resultant brown solid dried *in vacuo.* To this was added *N*-dimethylphenylenediamine (1.41 g, 10.3 mmol), MeCN (20 cm^3) and triethylamine (2.8 cm^3). The solution was refluxed under Ar for 22 h then evaporated to an orange oil which was chromatographed on alumina (Brockman grade II–III) eluted with hexane–ethyl acetate (10 : 1) to afford the product as a deep orange solid (587 mg, 71%). R_f 0.61 (alumina, hexane–EtOAc 10 : 1).

The NMR spectrum at room temperature in $CDCl_3$ exhibits more than one set of resonances, which are ascribed to conformers or aggregates. A single species was observed in DMSO at elevated temperature.

(Found: C 75.1; H 6.3; N 10.5. Calc. for $C_{25}H_{25}N_3O_2$: C 75.2; H 6.3; N 10.5%); λ_{max}(MeCN)/nm 408 (ε/dm^3 mol^{-1} cm^{-1} 11 000) nm; δ_H(400 MHz; DMSO; Me_4Si; 135 °C) 10.80 (1 H, br s, NH), 8.25 (1 H, d, *J* 7, Acr), 8.08 (1 H, d, *J* 6, Acr), 7.42 (1 H, t, *J* 7, Acr), 7.32 (1 H, br, Acr), 6.98 (1 H, t, *J* 7, Acr), 6.89 (1 H, br, Acr), 6.75 (2 H, d, *J* 9, Ar), 6.67 (2 H, d, *J* 9, Ar), 5.30 (1 H, sp, *J* 6, OCH), 2.93 (6 H, s, NCH_3), 1.43 (6 H, d, *J* 6, CH_3); δ_C(101 MHz; $CDCl_3$; Me_4Si) 167.88, 167.72, 151.28, 146.74, 143.78, 143.41, 140.94, 140.33, 137.97, 135.33, 133.56, 133.02, 131.04, 129.28, 126.48, 124.11, 123.04, 122.27, 120.12, 119.63, 119.23, 117.49, 116.65, 116.11, 114.75, 113.43, 112.58, 68.86, 67.96, 41.44, 25.61, 21.94; *m/z* (ES) 400.2031 ($C_{25}H_{26}N_3O_2$ requires 400.2020).

9-(4-Dimethylamino-phenylamino)-acridine-4-carboxylic acid. To a mixture of 9-(4-dimethylamino-phenylamino)-acridine-4-carboxylic acid isopropyl ester (525 mg, 1.31 mmol), THF (4 cm^3) and water (2.5 cm^3) was added $LiOH{\cdot}H_2O$ (110 mg, 2.62 mmol). After stirring at room temperature for 2 d, further portions of $LiOH{\cdot}H_2O$ (220 mg), THF (8 cm^3) and water (2 cm^3) were added and the mixture stirred at 40 °C overnight at which point tlc (alumina, hexane–EtOAc 4 : 1) indicated the absence of starting material. THF was removed under reduced pressure and the remaining solution diluted with water (50 cm^3), which was neutralized by dropwise addition of glacial acetic acid to afford a deep red precipitate which was collected by filtration. The

filtrate was extracted with $CHCl_3$ (2×25 cm^3 with several drops MeOH). The precipitate was solubilized in a mixture of $CHCl_3$ and MeOH, filtered, and combined with the $CHCl_3$ extracts, which were dried ($MgSO_4$) and evaporated to a brown solid. Column chromatography on silica gel eluted with $CHCl_3$–MeOH (9 : 1) afforded the product as a dark red powder (412 mg, 88%). R_f 0.48 (silica, $CHCl_3$–MeOH 9 : 1).

LCMS (Waters Atlantis C18, 4.6×30 mm, 1 cm^3 min^{-1}, 0–42 s 100% solvent A, with a gradient reaching 100% solvent B at 252 s, then 252–480 s 100% solvent B, where solvent A = aqueous 10 mM NH_4OAc with 0.1% formic acid, solvent B = 95 : 5 MeCN–H_2O): r.t. 3.50 min (m/z +358).

δ_H (400 MHz; DMSO; Me_4Si; 80 °C) 8.47 (1 H, d, *J* 7, Acr), 8.34 (1 H, d, *J* 8, Acr), 8.15 (1 H, br d, *J* 9, Acr), 7.84 (1 H, br d, *J* 8, Acr), 7.76 (1 H, t, *J* 8, Acr), 7.29 (2 H, m, Acr), 7.06 (2 H, d, *J* 9, Ar), 6.77 (2 H, d, *J* 9, Ar), 2.93 (6 H, s, NCH_3); m/z (ES) 358.1564 ($C_{22}H_{20}N_3O_2$ requires 358.1556).

Library synthesis

Polyamide synthesis was performed using standard Fmoc solid phase protocols on macrobead resin (Rapp, RinkAM, 0.6 mmol g^{-1}, ~100 nmole per bead). Side chain protecting groups were Arg(Pbf), Tyr(tBu), His(Trt) and Lys(Boc). The branch point was introduced using FmocLys(Fmoc)OH. Double couplings were employed for all residues. Variation was introduced into the N-terminal four residues using the split-and-pool method where X^1 = Tyr, Trp, Phe, Acr, X^2 = Arg, Im, Lys, X^3 = His, Lys, Py and X^4 = Arg, Val, Tyr. The beads were not recombined after the final coupling stage so that the identity of the N-terminal residue was known. Library beads were dispensed individually into wells of 96 well polypropylene plates and 100 μL cleavage reagent (95% TFA, 2.5% H_2O, 2.5% triisopropylsilane) was added. Plates were covered and allowed to stand for 6 h at rt, after which the cleavage reagent was evaporated by blowing with a stream of N_2, followed by drying *in vacuo* over KOH pellets.

Resynthesis of polyamides

Polyamides were prepared using standard Fmoc protocols on Rink amide resin (Novabiochem 100–200 mesh, 0.57 mmol g^{-1}) using PyBOP or HBTU for couplings. For branched polyamides, double couplings were employed after the branch point. Compound **B0** consists of the tri-glycine spacers, branch point and biotin tag. Cleavage was performed for 4 h with a mixture of 95% TFA, 2% *m*-cresol, 2% H_2O, 1% ethanedithiol, followed by precipitation with Et_2O. Compounds were purified by HPLC on a Phenomenex C18 Luna column and characterized by ES or MALDI MS and amino acid analysis (Department of Biochemistry, University of Cambridge). Concentrations of stock solutions were determined using amino acid analysis performed in duplicate with a norleucine standard.

ELISA screening

Phosphate buffer was used throughout (50 mM potassium phosphate, 100 mM KCl, pH 7.4). Streptavidin coated microplates (Streptawell Highbind 96 well, Roche Diagnostics) were incubated with annealed Htelo and Bck biotinylated oligonucleotides in adjacent wells (300 μL per well, 50 nM DNA in buffer) for 6 h at rt. Plates were washed (×4) with buffer.

Water (100 μL) was added to the crude cleaved polyamides and agitated gently for 30 min to give a stock solution of 1 mM nominal concentration based on bead loading. 5.5 μL of each stock was diluted to 110 μL with buffer, and 50 μL of this applied to adjacent wells (Htelo, Bck) of the DNA coated microplate. After incubation for 1 h at rt, plates were washed (×4) with buffer. Streptavidin-peroxidase polymer (Sigma) (1 : 10 000 dilution in buffer) was added to each well (50 μL), except for a pair of wells to which buffer alone (50 μL) was added. After incubation for 1 h at rt, the plate was washed (×4) with buffer containing 0.05% Tween 20 (Sigma). TMB substrate (Amersham) (100 μL) was added to all wells using a multichannel pipettor, followed by quenching after 3 min with H_2SO_4 (0.19 M, 100 μL). The absorbance was read immediately at 450 nm using a Biokinetics plate reader. The absorbance of the pair of wells left untreated with streptavidin-HRP was subtracted from the respective Htelo and Bck data points.

ELISA titrations

Phosphate buffer was used throughout (50 mM potassium phosphate, 100 mM KCl, pH 7.4). Streptavidin coated plates (96 well Streptawell Highbind, Roche) were incubated with Htelo (25 nM in buffer, 300 μL per well) for 6 h at rt. The plate was washed with buffer (×4). All wells were blocked with compound **B0** (1 μM in buffer, 100 μL) for 1 h at rt, followed by washing (×4) with buffer. Plate rows (columns 1–11) were incubated with serial dilutions of compounds **B1–B8** in buffer (50 μL), with buffer (50 μL) only in column 12. After 1 h, the plate was washed (×4), streptavidin-peroxidase polymer (50 μL, 1 : 20 000 dilution, Sigma) was added to all wells followed by incubation for 1 h at rt. Following washing with buffer (0.05% Tween 20), TMB substrate (100 μL, Amersham) was added to all wells using a multichannel pipettor. On development of a blue colour (several minutes) the reaction was quenched by addition of H_2SO_4 (100 μL). The absorbance was read immediately at 450 nm using a Biokinetics platereader. Data were fitted using a Langmuir model (eqn (1)) using the Solver module of Excel to determine an apparent association constant. A is the absorbance at 450 nm, K_a is the apparent association constant, and c is the polyamide concentration. The results, presented as $\log(K_a)$, are the mean of three determinations.

$$A = \frac{A_{max} K_a c}{1 + K_a c} \quad (1)$$

Surface plasmon resonance (SPR)

Surface plasmon resonance was performed on a Biacore 2000 instrument, using degassed phosphate running buffer (50 mM potassium phosphate, 100 mM KCl, pH 7.4). Sensor chips (Type SA, Biacore) were loaded with approximately 400 RU of Htelo and Dup. Eight serial dilutions of compound were injected at a flow rate of 20 μL min^{-1} and the equilibrium response determined relative to the baseline. The maximum compound concentrations were 10 and 100 μM for branched and linear polyamides respectively. Between injections the sensor surface was refreshed with injections of 1 M KCl and buffer. For branched polyamides, duplicate measurements were made on each of two different sensor chips, to give a total of four determinations

at each polyamide concentration. For linear peptides, duplicate measurements were made on a single sensor chip. After subtraction of the background response from the blank flowcell, the responses were normalized by division by R_{max}, the estimated response for a 1 : 1 DNA–polyamide complex, given by eqn (2). R_{DNA} is the loading of the DNA, M_{pept} and M_{DNA} are the molecular weights of the polyamide and DNA respectively.

$$R_{max} = \frac{R_{DNA} M_{pept}}{M_{DNA}} \quad (2)$$

An apparent association constant, K_a, was estimated as the gradient of a linear fit of R/R_{max} *versus* polyamide concentration using eqn (3) which is a simplification of the Langmuir equation when $K_a c \ll 1$. The value of R_{max} was calculated for a 1 : 1 polyamide–DNA complex from the measured DNA loading of the chip and the molecular weights of the polyamide and DNA.

$$R \approx R_{max} K_a c \quad (3)$$

Where no response is observed, an upper bound for the association constant is estimated as $\log(K_a) < 3.7$ based on the K_a that would be required to obtain a signal above the detection limit (2 RU) of the Biacore at the highest polyamide concentration used in the experiments.

Fluorescence resonance energy transfer quadruplex melts

HPLC purified and desalted oligonucleotide F21T was supplied by Eurogentec. The oligonucleotide at a concentration of 400 nM was annealed in buffer (50 mM KCl, 10 mM cacodylic acid, adjusted to pH 7.4 by KOH addition) by heating to 92 °C for 2 min and allowing to cool to room temperature over 4 h after which it was further diluted to 317 nM with buffer. Stock solutions of the branched polyamides **P1** to **P8** at concentrations of 42.9 μM were prepared in potassium chloride-cacodylate buffer. The stock oligonucleotide and polyamide solutions were supplied to a Gilson Cyberlab C-200 Robotic Workstation programmed to prepare 96 well plates (Bio-Rad MLL9651) containing 100 μL aliquots per well with an oligonucleotide concentration of 200 nM and a concentration series of 0, 0.10, 0.16, 0.25, 0.40, 0.63, 1.00, 1.59, 2.51, 3.98, 6.31 and 10.00 μM for each polyamide. The wells were covered (Bio-Rad TCS0803 Flat Cap Strips) and the plate placed in a DNA Engine Opticon 1 real-time PCR cycler (MJ Research). Fluorescence was measured at 0.5 °C intervals whilst increasing the temperature from 30 to 100 °C at a rate of 1 °C min^{-1}. Excitation was at 450–495 nm and detection at 515–545 nm. Data analysis was performed using Origin Pro 7.0 (OriginLab Corporation). A Labtalk script was written to import an ASCII file of the data into a worksheet and calculate the T_m from the maximum of the first derivative for each well. The T_m values were then plotted against concentration for each polyamide. Four repeat experiments were performed and the data from the four T_m–concentration plots were averaged to give the final plot shown. Kendall's rank correlation coefficient between ΔT_m and $\log(K_a)$ was calculated using the program kendl1.[20]

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