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Inhibition of Heat Shock Transcription Factor Binding by a Linear Polyamide Binding in an Unusual 1:1 Mode

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Abstract

Heat shock proteins (HSPs) are known to protect cells from heat, oxidative stress, and the cytotoxic effects of drugs, and thus can enhance cancer cell survival. As a result, HSPs are a newly emerging class of protein targets for chemotherapy. Among the various HSPs, the HSP70 family is the most highly conserved and prevalent. Herein we describe the development of a β -alanine rich linear polyamide that binds the GGA heat shock elements (HSEs) 3 and 4 in the HSP70 promoter in an unusual 1:1 mode and inhibits heat shock transcription factor 1 (HSF1) binding in vitro.

Keywords

polyamide; DNA; heat shock protein; transcription factor; inhibition; binding

Introduction

Heat shock proteins (HSPs)^[a] are part of a stimuli induced mechanism that protects cells from a wide range of intra- and extra-cellular environmental challenges including oxidative stress, drug toxicity, ischemia/reperfusion injury as well as heat. ^[1–5] Heat shock proteins can act as chaperones for the folding of newly synthesized proteins, assembly of multi-protein complexes, and the refolding or proteosomal degradation of denatured/damaged proteins. Among heat shock proteins, the 70 kD family members (HSP70) are the most highly conserved in protein sequence and also the most prevalent, being found in all organisms from *E. coli* to humans. ^[6, 7] Three of the HSP70 family of proteins are heat inducible, two of which are 99% homologous and collectively known as HSP70 as well as HSP72 and HSP70-1. ^[8]

In addition to its protective effects, HSP70 can enhance cancer cell survival through a number of mechanisms ^[9] such as by inhibiting cell apoptosis by both caspase-dependent and independent mechanisms, ^[10] as well as by stabilizing lysosomes. ^[11] Expression of HSP70 also significantly down-regulates the activity of NF- κ B ^[12] and inflammatory responses which might otherwise result in cell death, as evidenced by the increased sensitivity of HSP70 (HSPA1A and HSPA1B genes) -knockout mice to sepsis -induced death. ^[13] Loss of HSP70 in mice not only increases sensitivity to necrosis and

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inflammation, but also increases genomic instability and enhances radiosensitivity.^[6] Transformed cells often over-express HSP70 and depletion of these endogenous HSP70 levels induces cell death.^[14, 15] Given the ability of heat shock proteins to interact with a wide range of specific proteins of various signaling pathways and their essential role in maintaining cell survival, a much higher level of these proteins is needed for tumor cells to achieve accelerated metabolism required for rapid reproduction. HSP70 proteins are therefore emerging as promising targets for cancer therapy.^[16–18]

There are a number of inhibitors of HSP70 induction that have potential as chemotherapeutic agents and function by either directly inhibiting HSP70,^[19, 20] or by inhibiting transcription of the HSP70 gene.^[21] Direct inhibitors of HSP70 either target the N-terminal ATPase domain such as VER-155008^[22] and methylene blue^[23], or target the C-terminal substrate-binding domain such as 2-phenylethyne sulfonamide (PES).^[24, 25] These inhibitors suffer from either high IC₅₀ values or poor specificity, thus limiting their usefulness as HSP70 inhibitors.

Inhibitors that function by inhibiting transcription of HSP70 target the heat shock transcription factor 1 (HSF-1) which binds as a trimer to the heat shock elements (HSE) of HSP gene promoter.^[26] The most potent of these inhibitors is the diterpenoid triptolide (triptolide), which induces pancreatic cancer cell death *in vitro* and *in vivo* via inhibition of HSP70 expression^[27] by interfering with the heat shock factor transactivation process.^[28] Unfortunately, triptolide has severe toxic side effects in animals and humans, and its structural complexity does not make it an attractive target for further synthetic development. The much simpler synthetic compound KNK437 has also been shown to inhibit HSP70, but appears to require fairly high concentrations (100 µM) to be effective in cell culture.^[29] Another known inhibitor of HSP70 induction, quercetin, appears to work by inhibiting CK2 and CaMK2 catalyzed phosphorylation of HSF1.^[30] Quercetin also requires high concentrations and, though it is known not to be toxic in humans, lacks specificity, inhibiting many off-target kinases and enzymes.^[31, 32]

Another approach to HSP70 inhibitors would be to make use of highly programmable gene-specific agents, such as antisense, siRNA, and antigene agents.^[33] Among these potential agents, nucleic acid-based agents such as antisense phosphorothioates, locked nucleic acids, siRNAs, and peptide nucleic acids can be made to predictably bind to their nucleic acid targets by simple Watson Crick base pairing.^[33, 34] Unfortunately, all of these nucleic acid-based agents have poor membrane permeability, necessitating the use of liposomal, cell penetrating peptide, or nanoparticle delivery systems.^[33] Anti-gene agents based on polyamides can also recognize a target DNA sequence through predictable H-bonding interactions with the minor groove (Scheme 1), but unlike antisense agents, have better membrane permeability properties.^[35, 36] Minor groove binding polyamides have been shown to inhibit transcription factor binding to the major groove by modifications that block protein contacts with an adjacent major groove and/or DNA backbone,^[37, 38] or through allosteric effects.^[37, 39–43] Polyamides are also attractive for probe and drug developments, because libraries of compounds can be conveniently synthesized by standard solid phase peptide synthesis method using Fmoc building blocks.^[44] Herein, we report on the synthesis and binding properties of a series of hairpin and linear polyamides targeted to the human heat shock elements, and demonstrate for the first time, inhibition of transcription factor binding by a linear polyamide binding to DNA in an unusual 1:1 mode.

Results

The consensus sequence of the heat shock element (HSE) consists of multiple inverted repeats of nGAAn that are recognized by the heat shock transcription factor, HSF-1. There

are three heat inducible HSP70 genes in humans, of which HSPA1A and HSPA1B are the most conserved and bear the same promoter structure. [45] Promoters of the HSPA1A and HSPA1B genes contain three copies of the consensus sequence and two variants, nGACn and nGGGn (Figure 1A). [46] Two of the consensus sequences (site 3 and 4) form a pallindromic sequence with dyad symmetry (GGAATATTCC). HSF-1 binds as a trimer to the heat shock promoter and both footprinting and single nucleotide deletion analysis have shown that it preferentially binds to sites 3 and 4. [47] Based on methidiumpropyl-EDTA (MPE) footprinting results, it was proposed that purine tracts at site 1 and 3 have highly twisted DNA strands and narrowed minor groove, which help the heat shock transcription factor contact the major groove, but may make it difficult to target with a polyamide.

Hairpin polyamide design and synthesis

Recognizing that HSE3 and HSE4 are part of a pallindromic site, we initially decided to target the GGAAT sequence with a hairpin polyamide, as a hairpin polyamide has been previously successfully used to target the related GGAAA sequence contained in the NF- κ B binding site. [39] To this end we synthesized hairpin polyamides H0-H3 (Figure 1B). H1 was composed of only imidazole and pyrrole amino acids, whereas for H2 and H3 we replaced one pair of the pyrrole amino acids with β -alanine to relieve any potential strain induced by the site. β -alanine is preferred relative to pyrrole in structurally rigid DNA sites because of its flexibility. [48] H0 was used as a mismatch control for H1. The hairpins were synthesized in yields of 10%–20% by conventional solid phase Fmoc-synthesis [44] on an oxime resin. [49, 50]

Binding sites and affinity of the hairpin polyamides

The binding site and affinity of the hairpin polyamides for the HSE sites were determined by quantitative DNase I footprinting analysis. [51] To this end 5'-radiolabelled 152-mer DNA prepared by PCR containing the heat shock promoter was incubated with various concentrations of polyamide and subjected to DNase I cleavage. The extent of inhibition of DNase I cleavage at the observed binding sites as a function of polyamide concentration was then fit to a Hill-slope equation. DNase I footprinting showed that only H1 bound specifically to the DNA at concentrations below 1 μ M and at two distinct sites (Figure 2). One footprint encompassed HSE1, HSE2 and HSE3, but surprisingly, not HSE4, which contains the target GGAA sequence. HSE1 and HSE2 share a GGTT site between them that could also be bound by H1. The site higher up on the gel (E) corresponds to a site that also contains a GGAA site. Quantitative footprinting over the range of 100 to 500 nM gave the best fit for a fixed Hill slope of 1 with a binding constant of only $3.0 \pm 0.4 \times 10^5 \text{ M}^{-1}$ for the HSE1–4 sites. The observed binding affinity is a much lower than that of $1.3 \pm 0.2 \times 10^9 \text{ M}^{-1}$ that was reported for the corresponding GGAA site in the NF- κ B site. [39] Because this polyamide had such a low binding affinity and did not show any inhibition of HSF1 binding to the HSE (data not shown), we decided to investigate linear binding modes.

Linear polyamide design and synthesis

It has been found that a GGAA sequence in a quasi pallindromic site (AGGGAATCCCCT) can also be bound by linear polyamides in a 2:1, N $\rightarrow$ C vs 5' $\rightarrow$ 3' mode. [52] Recognition of this site was achieved with two four-ring-polyamides linked by β -alanine to enable binding to the long site. We therefore synthesized a library of linear polyamides based on ImImPyPyPyPyPy- β -Dp (P1) that were designed to bind in a 2:1 mode to HSE3/4 and incorporate β -alanine for increased flexibility. In one set of polyamides (P1–P5) each pyrrole of P1 except the last one was separately substituted with a β -alanine to help relieve potential stress induced by the HSE sequence (Figure 1C). In a second set, an additional pyrrole was substituted with β -alanine (P6 and P7), so that no two β -alanines were adjacent to each other. A double mismatch polyamide, P8, was also synthesized as a control.

A second library consisting of P9–P11 was designed to bind in a 1:1 mode in the C→N direction relative to the 5'→3' direction of the GG-containing strand of the target sequence that has been observed to be preferred for sequences containing multiple G's. [48, 53] The sequences of the second library were based on the P6 sequence, after it was found to have the highest affinity among the members of the first library and bind in a 1:1 mode by quantitative footprinting (Figure 3, *vide infra*). In addition, the recognition site of the polyamides were expanded to recognize the T•A bp to the 5'-side of the palindromic sequence on the top strand (position -6) in order to enforce a unique orientation that might be detectable by footprinting (Figure 1C). Binding in the opposite orientation was expected to be disfavored because there is C•G at the corresponding position (+6) in the sequence (underlined G in bottom strand in Figure 1C). A double mismatch polyamide P12 was also synthesized as a control. All the linear polyamides were synthesized by solid phase Fmoc synthesis [44] and a yield of around 40% was obtained for P1, but lower yields were obtained for the polyamides with additional β -alanine building blocks. P2–P5 and P9 were obtained in yields between 20% and 30%, while P6–P8 and P10–P12 were obtained in yields between 10% and 20%.

Binding sites and affinity of the linear polyamides

The binding sites and affinity of the linear polyamides were also determined by quantitative DNase I footprinting in the same way as for the hairpin polyamides. Members of the first library of linear polyamides having one or no β -alanines in the body portion (P1–P5) were determined to be the weakest binders as they did not show any readily discernable footprint at concentrations up to 500 nM (Figure 3A). Polyamides consisting of two β -alanines (P6 and P7) in the body portion showed a detectable footprint at the HSE3/4 site at 100 nM polyamide (Figure 3B). The mismatch polyamide P8, in which Im and β were switched, showed no distinct inhibition until 500 nM, at which point binding was fairly non specific (Figure 3C). Association constants determined by quantitative analysis of the footprinting gels (Table 1), show within the limits of error, that the 8-mer polyamides P6 and P7 binding in the N→C direction relative to the 5'→3' DNA direction have K_a 's of approximately $1 \times 10^7 \text{ M}^{-1}$. Whereas P7 appeared to bind in the expected 2:1 mode (Hill slope coefficient of 1.5), P6 appeared to bind in a 1:1 mode (Hill slope coefficient of 0.9).

Significantly higher binding affinity was observed for the second library of polyamides, P9–P11, which were designed to bind in the opposite direction (C→N) with respect to the GG-containing strand of the target sequence and to recognize the T to the 5'-side of HSE3 as shown in Figure 4. Whereas a footprint only began to emerge at about 100 nM for P9, much like for P6 and P7, a footprint began to emerge at 0.1 nM for the longer polyamides, P10 and P11. Quantitative analysis of the footprints (see Figure S1 for analysis of P10 and P11 over a narrower concentration range) shows that polyamides P9 – P11 have much higher affinity per amino acid than those binding in the N→C direction, with K_a 's of $1.15 \times 10^7 \text{ M}^{-1}$ for the 6-mer P9 to $3.44 \times 10^8 \text{ M}^{-1}$ for the 9-mer P11 (Table 1). Within this group of polyamides, the binding affinity increases with increasing length, in the order $P9 \ll P10 < P11$. Binding to non-targeted sites was observed to occur at the 3'-end of the promoter sequence for P10 and P11, but only at 100 nM and higher concentrations. The mismatch control for P11, polyamide P12, only bound to DNA substrate non-specifically at concentrations of 400 nM and above (Figure 4). All of the C→N binding polyamides appeared to bind in a 1:1 mode based on the Hill Slope coefficient (Table 1).

Although DNase I footprinting is not able to determine the exact bases bound by a polyamide, or the orientation of the polyamide, close examination of the footprints for P9–P11 reveals an increase in the size of the protected site with an increase in the size of the polyamide (Figure 5). Comparison of the blocked sites in the bracketed section of the gel indicates that the relative intensity of one cleavage band is greatly diminished in going from

P9 to P10, and that then next band is lost in going from P10 to P11. Thus, increasing the polyamide chain length at the N-terminus increased its DNA binding and hindered DNase I accessibility to the bound DNA. The loss of DNase I bands on going from P9 to P10 to P11 is most consistent with binding in the C→N orientation relative to the 5'→3' direction of the top strand, though we cannot rule out that some binding is also occurring in the opposite orientation.

In vitro inhibition of HSF-1 binding to heat shock element DNA by polyamides

The ability of the polyamides to block binding of HSF-1 to its cognate HSE sequence was determined by a standard electrophoretic mobility shift assay with a duplex ODN and cell extracts. [56, 57] Thus, 3'-end labeled HSE 36-mer duplex (10 nM) was incubated with 100 nM polyamide for 16 h at 25 °C and then mixed with a nuclear extract prepared from heated Jurkat cells. The formation of the HSE/HSF-1 complex (indicated by the arrow) was then assayed by polyacrylamide gel electrophoresis and quantified by comparison to the unshifted band (Figure 6). As expected, the shifted HSE/HSF-1 complex readily formed with nuclear extracts prepared from heated cells in the absence of added polyamide, and no shifted band was observed with unheated extracts. Addition of polyamides P3–P6, P8–10, or P12 to the HSE binding reactions did not significantly decrease the amount of shifted complex, and so was the case for P1–P2 or P7 (data not shown). Polyamide P11, however, reproducibly decreased the amount of HSE/HSF-1 complex to about 35% of control, indicating that polyamide binding inhibited HSF-1 binding to the HSE. The electromobility shift assay was then carried out with different concentrations of P11 in comparison to P8 and P12, to determine its minimum inhibitory concentration (Figure 7). Whereas P8 had no effect on complex formation over a range of 10 to 100 nM, the P11 polyamide inhibited complex formation at 50 and 100 nM but not at lower concentrations (Figure 7A). Similar observations were made for P11 versus P12 (Figure 7B).

Discussion

There are several examples of hairpin or cyclic polyamides that inhibit transcription factor binding to the DNA major groove, presumably by altering the minor groove conformation and as a consequence the major groove conformation. [40, 42, 43, 58] There are some examples of linear polyamides binding in a 1:1 mode that can alleviate inhibition of transcription in expanded GAA•TTC repeats, by stabilizing a B DNA conformation and interfering with intramolecular triplex formation and intermolecular interactions. [59] There is also evidence that linear polyamides binding in a 1:1 mode are able to displace proteins from heterochromatin. [60, 61] Until our study, there have not been, to our knowledge, any reports of unmodified linear polyamides binding in a 1:1 mode that can inhibit transcription factor binding.

Our initial attempts to design hairpin polyamides H1–H3 to bind to the GGAA sequence in HSE3 and HSE4 along previously successful lines for a similar site in the NF-κB site, [39] only yielded a weak binding polyamide H1 that was not able to inhibit heat shock transcription factor binding *in vitro* (data not shown). In this case, introduction of β-alanines in place of pyrroles in the parent H1 sequence to produce H2 and H3 had a negative effect on binding. H1 also did not appear to be very specific, as footprinting indicated that it was recognizing GGAA or GGTT sites in HSE1, HSE2, HSE3 and site E, but surprisingly not the GGAA site in HSE4. The weak binding of H1 may be due to a narrower minor groove adopted by HSE, and it was for this reason that we turned our attention to linear polyamides.

Our first design of a linear polyamide, P1, that was expected to bind in a 2:1 mode made use of only internal pyrrole and imidazole building blocks but failed to bind to the HSE significantly, possibly because of the distorted nature of the heat shock element. When

pyrroles were replaced with the more flexible β -alanine units, [48] only polyamides containing two internal β -alanines, P6 and P7, bound with significant affinity to the targeted HSE3 and HSE4. Due to the pallindromic nature of this sequence, P6 and P7 could be binding in an N $\rightarrow$ C orientation to either strand in a 1:1 mode, [53] or both strands in a 2:1 mode. [62] While the Hill slope coefficient for P7 (1.53 ± 0.18) indicated binding in a 2:1 mode, the coefficient for P6 (0.89 ± 0.18) was consistent with a 1:1 binding mode, the (Table 1).

Because HSE3/4 contains GGAA sequences, it was also possible that polyamides could be made to bind in the 1:1 C $\rightarrow$ N mode vs the 5' $\rightarrow$ 3' direction of the GG-containing strand of the target sequence. [48, 53] In support of this idea, polyamide P11, which was designed to bind in this mode to the GGAA of HSE3 by recognizing the T at the -6 position, had significantly higher binding affinity than the sequences designed to bind in the 2:1, N $\rightarrow$ C vs 5' $\rightarrow$ 3' mode. Also consistent with the 1:1 C $\rightarrow$ N binding mode of P11 was the increase in the DNase I footprint on going from P9 to P10 to P11 (Figure 5) as well as the Hill slope coefficient of 0.86. In the case of the heat shock elements, the presence of two opposing polypurine tracts interrupted by a TA sequence may allow binding of polyamides in both N $\rightarrow$ C and C $\rightarrow$ N orientations.

It is noteworthy that while a GGAA sequence in the NF-kB binding site or in a quasipallindromic site could be bound with high affinity by a hairpin polyamide [39] or by a linear polyamide in a 2:1 N $\rightarrow$ C mode, [52] respectively, the GGAA sequence in the HSE3/4 sequence appears to prefer binding to a linear polyamide in a 1:1 mode. It may be that the over-twisted nature of the sequence containing two HSE elements precludes binding of a second polyamide to the minor groove. Given the similar binding affinities (Table 1) and footprints (Figure 5) of P10 and P11, it was surprising that only P11 showed significant inhibition of HSF-1 binding to the HSE in an electrophoretic mobility shift assay (Figure 6). It may be that the ability of P11 to cover one additional base pair is just enough to block binding, either by changing the conformation of the DNA or by interfering with a phosphate contact. [39, 43]

Initial attempts to inhibit HSP70 induction in Jurkat cells by addition of P11 to the culture medium failed, suggesting that the polyamide was not entering the cell in high enough concentration to be effective. When the P11 and the mismatch control P8 were electroporated into Jurkat cells, an approximately 40% decrease in heat induced HSP70 protein synthesis was observed for P11, but not for P8 (Fig. S3), suggesting that P11 was able to block HSF-1 binding *in vivo*. Although a promising result, more studies will be required to validate this mechanism of inhibition. It may be that incorporation of the β -alanines hampered cell permeation of the polyamides as has found to be the case for other polyamides, [12, 63, 64] and that future studies will need to focus on improving cell delivery. It has been shown that cell permeability of polyamides can be enhanced by various modifications at the C-terminus such as with a short ethylene diamine tail, [65] an oxime-linkage between the C-terminus and an aromatic moiety, [66] dye-conjugation, [63, 64] and attachment to cell-penetrating peptides. [67]

Conclusion

We have demonstrated that polyamides with high affinity and specificity for a GGAA-containing heat shock element of the HSP70 promoter can be achieved through the use of linear β -alanine-polyamide binding in an unusual 1:1 mode with a C $\rightarrow$ N binding orientation relative to the GG-containing strand of the target sequence. We also showed that one of these linear polyamides can also inhibit heat shock transcription factor binding in cell lysates. To our best knowledge this is the first time that a linear polyamide binding in a 1:1

mode has been shown to block transcription factor binding, and should serve as a model for the design of polyamides targeted to other polypurine-rich promoter sequences. Though our polyamide showed good inhibition of heat shock transcription factor binding in cell lysates, preliminary studies indicate that it is not able to inhibit HSP70 induction in cell culture alone and requires electroporation to obtain an effect. Unfortunately, the multiple β -alanines used to obtain high affinity binding may have compromised the ability of the polyamide to penetrate into the cell and/or nucleus, and future work will need to focus on improving the cell permeability of this polyamide. Given that a considerable amount of heat shock protein may also be induced from the HSPA6 gene, polyamides targeting the HSPA6 heat shock elements may also be needed in conjunction with those for the HSPA1A/A1B elements, in order to completely suppress the heat shock response.

Experimental Section

Materials

Anhydrous dimethyl formamide was from EMD chemicals Inc. Anhydrous dichloromethane was freshly prepared by refluxing with calcium hydride followed by distillation. HATU was purchased from Genscript Corporation. All other reagents were directly purchased either from Omni Solvent or Sigma Aldrich unless otherwise specified. DNase I was from Invitrogen. Amicon centrifugal filters were from Millipore. Analytical thin-layer chromatography was performed on Aldrich Silica gel 60 F₂₅₄ plates (0.25 mm). Fmoc deprotection was qualitatively monitored by UVG-54 mineral light UV lamp at 254 nm.

Chemical synthesis of polyamides

Polyamide building blocks Fmoc-Im-COOH, Fmoc-Py-COOH, Fmoc- β -alanine and Im-COOH were synthesized according to published procedures as was the manual synthesis of polyamides with some modifications. [44] Synthesis of the hairpin polyamides was carried out on an oxime resin in plastic columns (Bio-Rad) that were shaken in an automatic shaker (Labnet International. Inc), [49, 50] while the synthesis of the linear polyamides was carried out on Fmoc- β -alanine-Wang's resin (200 mg, 0.34 mmol/g). Before synthesis, resins were immersed in a 1:1 DCM/DMF mixture for a few hours to allow sufficient time for swelling. One synthesis cycle was used for all couplings except when coupling to imidazole, when two synthesis cycles were used. One cycle of synthesis consisted of Fmoc deprotection followed by washing, coupling followed by washing, and finally a capping step followed by washing. The Fmoc deprotection step was carried out by mixing the resin with 20% piperidine in DMF and then shaking the mixture for 3 min. The deprotection solution was then drained and spotted on thin-layer chromatography plate to monitor the formation of the deprotected product, which turns to a blue or purple color under a UV 254 nm lamp. The column was then washed with DCM for 3 min, followed by DMF for 3 min, and then 20% piperidine was added again and shaken for additional 20 min. The washing step was carried out by mixing the resin with DCM for 3 min and then draining, then with DMF for 3 min and draining, and then repeating these two washes once again, followed by a final wash with DMF. The coupling step was carried out with DMF (700 μ L) plus DIPEA (300 μ L), containing a building block (0.136 mmol) and a molar equivalent of HATU (0.136 mmol). The building blocks were preincubated with HATU/DIPEA in DMF for 5 min immediately before adding to the resin. The mixture was shaken for 1 h when coupling pyrrole and 2 h when coupling imidazole, whereas at least 8 h were required when coupling any building block to imidazole. The capping step was carried out by shaking the resin with 5% pyridine and 20% acetic anhydride in DMF for 3 min, followed by draining and washing with DCM and DMF for 3 min each. The resin was then shaken for 20 min with additional capping reagent, followed by draining and washing. At the end of synthesis, polyamides were cleaved from resins (50 mg) by treatment with 2 mL of 1-dimethylaminopropylamine at

55°C, for 24 h. The 1-dimethylaminopropylamine solution was filtered and evaporated to afford a brownish residue that was triturated with diethyl ether. The yellow to white precipitates were purified by preparative reverse phase gradient HPLC on a Beckman System Gold HPLC system with a solvent pump module 125 and model 168 diode array UV detector, equipped with a reverse phase column XTerra Prep MS C18, (10 µm, 7.8 × 300 mm). HPLC was carried out with a linear gradient of 0 – 5 % solvent B (0.1 % TFA in acetonitrile) in solvent A (0.1% TFA in water) over 0.05 min, followed by 5 – 100% solvent B in solvent A over 50 min. The purified products were characterized by MALDI (Table S1), and were >95% pure as judged by analytical HPLC.

Preparation of the DNase I footprinting substrate and sequence ladders

The DNA substrate for the DNase I footprinting assays was prepared by polymerase chain reaction on an HSPA1A containing plasmid [68] with 5'-[³²P]-end labeled d(CACTCTGGCCTCTGATTGGT) and d(CCCTGGGCTTTTATAAGTCGT) to give the 5'-end-labeled 152-mer product d(CACTCTGGCCTCTGATTGGTCCAAGGAAGGCTGGGGGGCAGGACGGGAGGCG AAACCCCTGGAATATTCCTGACCTGGCAGCCTCATCGAGCTCGGTGATTGGCTC AGAAGGGAAAAGGCGGGTCTCCGTGACGACTTATAAAAGCCCAGGG) and its complementary strand. After PCR, the product was mixed with Promega blue/orange loading dye (6 ×) and purified according to the published procedure. [51] The Maxam-Gilbert A+G sequence ladder was prepared according to a standard procedure, [69] while the A sequence ladder was prepared according to another procedure. [70]

DNase I footprinting

DNase I footprinting of the polyamides was carried out according to a standard procedure, [51] except that the polyamide was incubated with the 152-mer DNA substrate for 24 h at room temperature, followed by the addition of an optimized amount of DNase I (approximately 0.025 mU/µL final conc.). The reaction was then quenched after 5.5 min, ethanol precipitated and electrophoresed on an 8% polyacrylamide denaturing gel (7 M urea). The gel was exposed to a storage phosphor screen and imaged with a Biorad Personal Molecular Imager.

Quantitative analysis of footprinting results

The intensity “I” of bands in the footprinting gels was quantified by Biorad Quantity One software. The fractional occupation θ by the polyamide was calculated as $1 - (I_{\text{site}}/I_{\text{ref}})/(I^{\circ}_{\text{site}}/I^{\circ}_{\text{ref}})$, where I_{site} is the intensity of a band within the binding site that is modulated by polyamide, I_{ref} is the intensity of a band outside the binding site that is not modulated by polyamide binding, and I°_{site} , I°_{ref} are the intensities of the same sites in the absence of polyamide. [51] The observed fractional occupation as a function of polyamide concentration (L) was then fit to a variable Hill slope (n) equation with Graph PadPrism 5 using nonlinear regression analysis (equation 1). K_a was obtained by equating EC_{50} with $1/K_a$ and averaging for three independent sets of measurements.

$$\theta = \theta_{\min} + \frac{(\theta_{\max} - \theta_{\min})}{1 + 10^{n(\log EC_{50} - L)}} \quad (1)$$

In vitro gel-shift assay of heat shock factor 1 binding

Gel mobility shift assays were performed essentially as previously described [30] using nuclear extracts prepared from control and heated (43 °C for 30 min, 1 h recovery at 37 °C) human Jurkat cells. Two complementary ODNs,

d(GTAGGCGAAACCCCTGGAATATTTCCCGACCTG) and d(CTGCCAGGTCGGGAATATTCCAGGGGTTTCG), derived from the human HSPA1A gene promoter that contained HSE 1–5 were synthesized and purified by polyacrylamide gel electrophoresis (Eurofins). After annealing, the 5'-overhanging ends of the duplex DNA were filled with α -³²P-dATP and cold nucleotides using Klenow polymerase to give a 36-mer radiolabelled duplex. Polyamides (10–500 nM) were bound to the labeled duplex ODNs (10 nM) by incubation together in annealing buffer (10 mM Tris pH 7.5, 10 mM NaCl, 1 mM EDTA) for 16 h at 25 °C. Mobility shift binding reactions containing nuclear proteins (1.5 µg) mixed with equivalent counts of labeled probe (25 µL final volume) were incubated for 20 min at 25 °C before separation on 4.5% polyacrylamide gels. Labeled protein-DNA bands were detected and quantified on a Molecular Dynamics PhosphorImager.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Design of polyamide libraries targeting the promoter region of DNA encoding for heat shock protein 70. A) The promoter region of the human heat shock protein A1A/A1B gene contains five adjacent heat shock elements (HSE), which binds to the trimerized form of heat shock transcription factor 1 (HSF1) in its major groove. HSE3 and HSE4 combine to form a palindromic site (boxed) with the dyad axis indicated with a filled diamond. B) Hairpin polyamides targeted to the minor groove of the GGAA sequence. C) Linear polyamide libraries designed to target the minor groove of heat shock elements HSE 3 and HSE 4 in 2:1 (P1–8) and 1:1 (P9–12) modes. The orientation of the polyamide (N→C or C→N) with respect to the 5'→3' direction of the top strand is shown to the right, and mismatches in the polyamide are underlined. Because of the palindromic nature of the boxed target site, polyamides P1–P8, can also bind equally well to the bottom strand in the reverse orientation with respect to how they would bind to the top strand to form a 2:1 complex. Because polyamides P9–P12 were designed to recognize the T•A bp at position -6 of the top strand with a β-alanine, binding in the opposite orientation would result in a mismatch between the underlined C•G bp at the 6 position and the β-alanine group and would be disfavored. The bottom strand is written in a 5'→3' direction and numbered according to the number of the complementary base in the top strand.

Figure 2.

DNase I footprinting of the hairpin polyamide library. A) Denaturing polyacrylamide gel for H0 that is representative of H2 and H3. The concentration of polyamide increased from left to right: 0, 10, 50, 100, 500 pM, 1, 5, 10, 50, 100, 500 nM, 1 μ M. B) The concentration of polyamide H1 increased from left to right: 0, 100, 200, 300, 400, 500 nM. C is a control with no polyamide or DNase I, A is the Maxam Gilbert A sequencing lane. Binding sites are labeled according to the scheme shown in panel c below. C) Sequence of the 152-mer (only 26–150 shown) with HSE sites indicated as numbers 1–5, as well as other sites observed to be bound by the hairpin and linear polyamide, as letters A–E. Antiparallel arrows indicate

that the nGnnn HSE sequence is in the opposite strand. The A's that appear in the sequencing lanes are underlined.

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Figure 3.

DNase I footprinting of the N→C linear polyamide library P1–P8. Polyamide concentration increases from left to right at 0, 0.1, 1, 10, 50, 100, 200, 300, 400 and 500 nM. Lane C is a control lane containing only DNA and 500 nM polyamide and no DNase I. Lane A is the Maxam Gilbert A sequencing reaction lane. Gel A) shows the footprinting of P1 which is representative of P2–P5. Gel B) illustrates the footprinting of P7 which is representative of P6, and gel C) is the result of the mismatch control P8. Binding sites are labeled according to the scheme shown in Figure 2C.

Figure 4.

DNase I footprinting of the C→N linear polyamide library. Polyamide concentration increases from left to right at 0, 0.1, 1, 10, 50, 100, 200, 300, 400 and 500 nM. Lane C is a control lane with DNA and 500 nM polyamide and no DNase I. Lane A is the Maxam Gilbert A sequencing reaction. Lane Ct is a control lane showing the cleavage products of DNA treated with DNaseI for 30 s, the other lanes are treated for 5.5 min. P12 is the mismatch control for P9–P11. Binding sites are labeled according to the scheme shown in Figure 2C.

Figure 5.

Analysis of the DNase I footprints of P9–P11 at HSE3 and HSE4. Because the HSE3/4 site is palindromic, the linear polyamides are shown aligned in C→N orientations with respect to the GG-containing strand of the target sequence. The mismatch G•C base pair is underlined. C is a control lane with DNase I in the absence of any polyamide. The bracket to the left indicates DNase cleavage sites that become progressively blocked on going from P9 to P11. To align the DNase I bands with the A sequencing lane, one has to take into account that the DNA is 5'-end-labeled and that DNase I cleaves to the 3'-side of a nucleotide producing a 5'-end-labeled product with a 3'-OH. The Maxam Gilbert A reaction, on the other hand, cleaves to the 5'-side of the nucleotide and produces a 5'-end-labeled product with a 3'-phosphate. Because the 3'-phosphate increases the mobility of a DNA fragment relative to a 3'-OH by about one nucleotide or more, [54, 55] the band corresponding to cleavage to the 3'-side of an A by DNase I corresponds to a band about 2 nt higher up on the gel than produced by a Maxam Gilbert A reaction.

Figure 6.

Electromobility-shift assay of the inhibition of HSF1 binding to the HSE by the polyamides. ^{32}P -radiolabeled HSE 36-mer (10 nM) was incubated with each polyamide (100 nM) for 16 h before mixing for 20 min with nuclear extracts (1.5 μg protein) prepared from heated Jurkat cells (43 $^{\circ}\text{C}$ for 30 min with 1h recovery at 37 $^{\circ}\text{C}$) and then electrophoresed on a non-denaturing polyacrylamide gel. The position of the HSE/HSF-1 complexes is indicated by the arrow. Control lanes: 36-mer radiolabeled HSE ODN alone (ODN), and in the presence of unheated (– heat) and heated (+ heat) Jurkat cell extract.

Figure 7.

Electromobility shift assay of the effective inhibitory concentration of polyamide P11 compared to mismatched P8 and P12. Polyamides were incubated at the indicated final concentrations with ^{32}P -radiolabeled HSE 36-mer duplex ODN for 16 h at 25 °C before mixing with heat shocked Jurkat cell nuclear extracts and then electrophoresis as described in Figure 6. The position of the HSE/HSF-1 complexes is indicated by the arrow. Control lanes: HSE 36-mer duplex in the absence of extract (ODN), and in the presence of unheated (– heat) and heated (+ heat) Jurkat cell extract.

Scheme 1.

Polyamide structure, binding, and synthetic intermediates. Polyamides are composed of N-methyl-imidazole (Im), N-methyl-pyrrole (Py) and β -alanine (β) that recognize DNA sequences through hydrogen bonding to the minor groove of Watson-Crick base pairs by side pairing of Im/Py for G/C, Py/Im for C/G, and Py/Py for A/T or T/A, respectively. The more flexible β/β can also recognize A/T or T/A. When present, an N-terminal imidazole group does not have an N-terminal amino functional group.

Table 1

Association constants and Hill slope coefficients for the linear polyamides.

Polyamide	Length	K_a (M^{-1})	Hill Slope coefficient
P6	8	$1.39 \pm 0.46 \times 10^7$	0.89 ± 0.18
P7	8	$8.84 \pm 0.35 \times 10^6$	1.53 ± 0.18
P9	6	$1.15 \pm 0.24 \times 10^7$	0.95 ± 0.28
P10	8	$1.19 \pm 0.90 \times 10^8$	0.68 ± 0.41
P11	9	$3.44 \pm 1.4 \times 10^8$	0.86 ± 0.42