Chapter 4B

Inhibition of VEGF with β-Alanine-C_3-Linked Hairpin Polyamide Conjugates

This project was done in collaboration with Nicholas Nickols (Dervan group, Caltech).

Nicholas Nickols synthesized polyamide **1**.

Abstract

Hairpin polyamide conjugates synthesized on Boc-β-Ala-PAM resin and cleaved with 1,3-diaminopropane have been observed to exhibit favorable nuclear uptake properties in human cancer cell lines. In this study, DNase I footprint titrations indicate that these small molecules bind the hypoxia response element 5'-TACGTG-3' with affinities of $K_a > 3 \times 10^9$ M^{-1}. Polyamide-fluorescein conjugates in this class of compounds traffic to the nucleus of live HeLa cells. A series of hairpin polyamide conjugates with the β-alanine-C_3 linker was assayed by quantitative RT-PCR for effects on hypoxia-inducible transcription of vascular endothelial growth factor (VEGF). Fluorescein and isophthalic acid β-alanine-C_3-linked polyamide conjugates **2**, **4**, and **5** are shown to decrease VEGF mRNA expression.

4.5. Introduction

Pyrrole-imidazole polyamides are synthetic ligands that bind the DNA minor groove at subnanomolar concentrations in a sequence-specific manner.[1,2] Polyamide-fluorophore conjugates have been imaged by confocal laser scanning microscopy to determine the extent of nuclear localization.[3-6] These experiments showed that the use of a fluorescein dye on the amine tail improved uptake.[4] Another trend that emerged was that conjugates containing the β-alanine linkage exhibited poor cellular uptake across a number of cell lines.[5,6] For instance, ImImPyPy-γ-ImPyPyPy-(+)-FITC localized to the nucleus of all cell lines tested, while ImImPyPy-γ-ImPyPyPy-β-(+)-FITC was excluded from the nucleus.[5]

However, these studies also included several examples of β-alanine-linked polyamide-fluorescein conjugates that traffic unaided to the nucleus.[5,6] These observations suggested that the composition of the tail linker could have a positive effect on uptake. Recent studies on polyamide-fluorescein conjugates synthesized with Boc-β-Ala-PAM resin using a 1,3-diaminopropane linker showed improved nuclear localization. Specifically, imaging experiments with β-alanine-C_3-linked FAM conjugates containing a WM moiety showed that these polyamides localized to the nucleus of HeLa, MCF-7, and PC3 human cancer cell lines.[7] Furthermore, in Chapter 4A, the hairpin polyamide conjugate ImImPyPy-(*R*)^{H_2N}γ-ImPyPyPy-β-C_3-FITC was observed to localize to the nucleus of the same three cell lines. Interestingly, in this case, the FAM conjugate showed poorer uptake. These promising examples indicate that an uncharged linker could allow the use of Boc-β-Ala-PAM resin in the synthesis of cell-permeable hairpin polyamide conjugates suitable for gene regulation.

A series of hairpin polyamide conjugates **2-5** was synthesized on Boc-β-Ala-PAM resin, and 1,3-diaminopropane was used to link a fluorescein or isophthalic acid moiety to the polyamide tail (Figure 4.5). The DNA binding affinity of these compounds for the hypoxia response element 5'-TACGTG-3' was determined by DNase I footprint titrations. Nuclear localization of the polyamide-fluorescein conjugates was imaged in HeLa cells. Targeting of polyamides to the hypoxia response element has been shown to disrupt the binding of

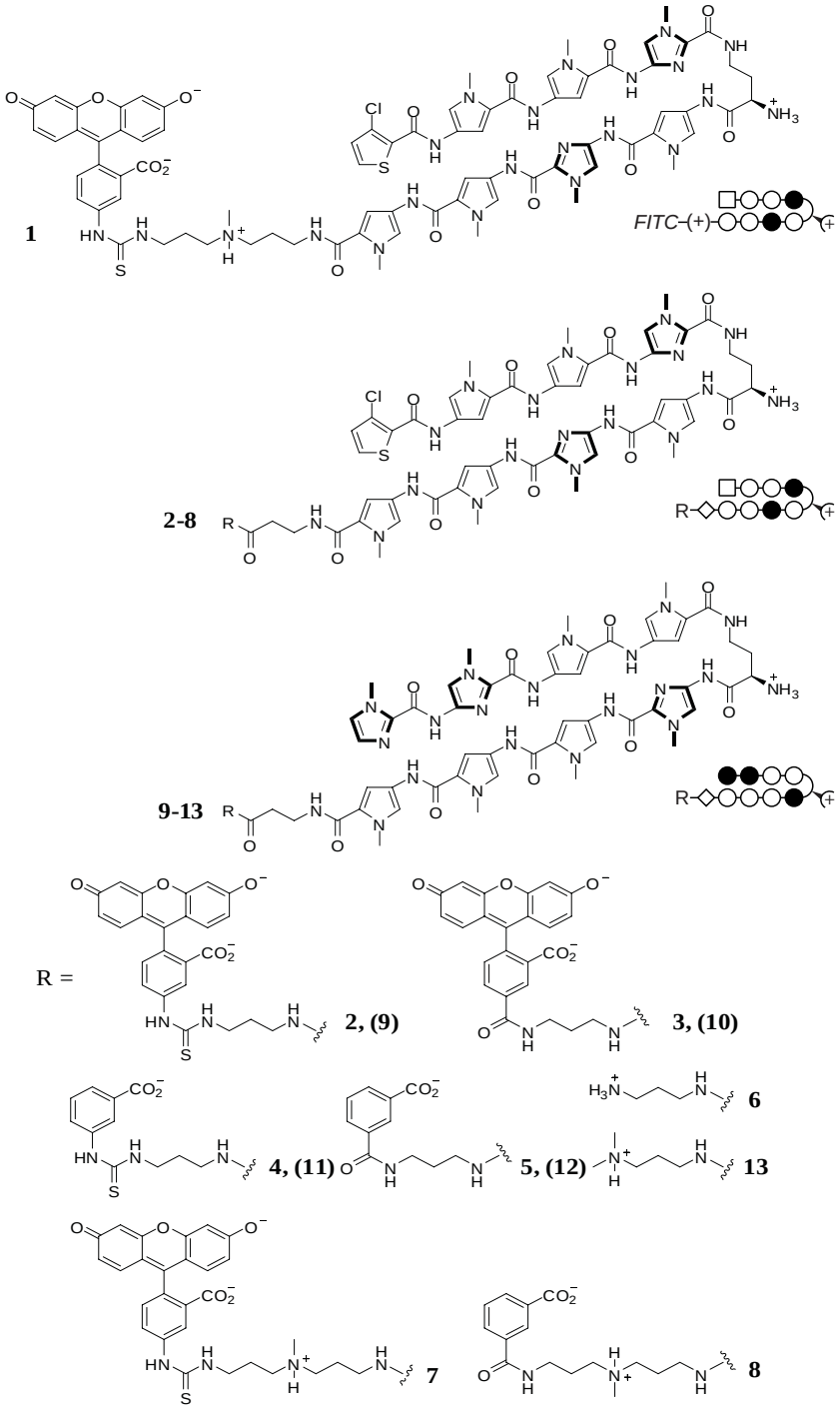

Figure 4.5. Structures of polyamide conjugates **1-13**. Imidazole and pyrrole are shown as filled and non-filled circles, respectively; chlorothiophene is shown as a square; β-alanine is shown as a diamond; the 1,3-diaminopropane linker is shown as "C_3"; the 3,3'-diamino-*N*-methyldipropylamine linker is shown as "(+)"; and the chiral diaminobutyric acid turn residue is shown as a semicircle connecting the two subunits linked to a half-circle with a plus.

hypoxia-inducible factor as measured by reduction in vascular endothelial growth factor (VEGF) gene expression.[8-10] The activity of β-alanine-C_3-linked polyamide conjugates **2**-**5** was assayed by quantitative RT-PCR to determine the level of VEGF mRNA transcript.

4.6. Materials and methods

4.6.1. Polyamide synthesis

Polyamides were synthesized with oxime resin (compound **1**, Novabiochem) or Boc-β-Ala-PAM resin (compounds **2**-**13**, Peptides International) according to published manual solid-phase synthesis protocols.[11,12] For compounds **1**-**13**, *N*-α-9-fluorenylmethoxycarbonyl-*N*-γ-*tert*-butoxycarbonyl-D-2,4-diaminobutyric acid (Fmoc-D-Dab(Boc)-OH, Peptides International) was used for the turn linkage. The protected ^{FmocHN}γ-turn amine was deprotected with 20% piperidine in DMF and reprotected as the Boc derivative with a solution of Boc_2O (Fluka) and DIEA in DMF. The Boc-protected resin was cleaved with 1 mL of the appropriate amine (3,3'-diamino-*N*-methyldipropylamine, compounds **1**, **7**, **8**; 1,3-diaminopropane, compounds **2**-**6**, **9**-**12**; 3-dimethylamino-1-propylamine, compound **13**) at 37°C with agitation for 16 h. Products were purified by preparatory reverse-phase high-performance liquid chromatography (HPLC) on a Beckman Gold system using either a Waters Delta-Pak 25 × 100 mm, 15 μm 300 Å C_{18} PrepPak Cartridge reverse-phase column or a Varian Dynamax 21.4 × 250 mm Microsorb 8 μm 300 Å C_8 reverse-phase column in 0.1% (w/v) TFA with acetonitrile as the eluent. The appropriate fractions were lyophilized after characterization by analytical HPLC, UV-visible spectroscopy, and matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF-MS) and/or electrospray ionization mass spectrometry (ESI-MS). HPLC analysis was performed on a Beckman Gold system equipped with a diode-array detector using a Phenomenex Gemini 4.6 mm × 250 mm, 5 μm 110 Å C_{18} reverse-phase column. UV spectra were measured on an Agilent Technologies 8453 UV-vis ChemStation spectrophotometer. MALDI-TOF-MS was carried out on an Applied Biosystems Voyager DE-PRO.

Conjugates were formed by reacting the isothiocyanate (compounds **1**, **2**, **4**, **7**, **9**, and **11**) or succinimidyl ester (compounds **3** and **10**) with the polyamide in a solution of DIEA (20 equiv) and DMF for 1 h at room temperature. Conjugates **5**, **8**, and **12** were formed by preactivating isophthalic acid (3.0 equiv) with PyBOP (2.9 equiv, Novabiochem) in a solution of DIEA (20 equiv) and DMF at 37°C for 30 min, followed by reaction of the activated solution with the polyamide for 1 h at room temperature. Conjugates were deprotected with neat TFA (Halocarbon) and triethylsilane for 30 min at room temperature before purification by preparatory reverse-phase HPLC. Lyophilization of the appropriate fractions yielded the polyamide conjugates **1**-**13**, which were characterized as described above. Extinction coefficients were calculated according to standard protocols.[13] Chemicals not otherwise specified were from Aldrich.

CtPyPyIm-(*R*)^{H_2N}γ-PyImPyPy-β-C_3-FITC (2). Cleaved from β-Ala-PAM resin with neat 1,3-diaminopropane and conjugated with fluorescein-5-isothiocyanate (FITC, Invitrogen). UV-vis λ_{max} = 315, 443 nm; ESI-MS *m*/*z* 1633.6 ($C_{76}H_{74}ClN_{22}O_{15}S_2^-$ calculated $[M-H]^-$ 1633.48)

CtPyPyIm-(*R*)^{H_2N}γ-PyImPyPy-β-C_3-FAM (3). Cleaved from β-Ala-PAM resin with neat 1,3-diaminopropane and conjugated with 5-carboxyfluorescein, succinimidyl ester (5-FAM, SE, Invitrogen). UV-vis λ_{max} = 313, 446 nm; MALDI-TOF-MS *m*/*z* 1605.40 ($C_{76}H_{75}ClN_{21}O_{16}S^+$ calculated $[M+H]^+$ 1604.51)

CtPyPyIm-(*R*)^{H_2N}γ-PyImPyPy-β-C_3-CPITC (4). Cleaved from β-Ala-PAM resin with neat 1,3-diaminopropane and conjugated with 3-carboxyphenyl isothiocyanate (Trans World Chemicals). UV-vis λ_{max} = 309 nm; ESI-MS *m*/*z* 1423.4 ($C_{63}H_{68}ClN_{22}O_{12}S_2^-$ calculated $[M-H]^-$ 1423.45)

CtPyPyIm-(*R*)^{H_2N}γ-PyImPyPy-β-C_3-IPA (5). Cleaved from β-Ala-PAM resin with neat 1,3-diaminopropane and conjugated with isophthalic acid. UV-vis λ_{max} = 314 nm; MALDI-TOF-MS *m*/*z* 1394.72 ($C_{63}H_{69}ClN_{21}O_{13}S^+$ calculated $[M+H]^+$ 1394.48)

CtPyPyIm-(*R*)^{H_2N}γ-PyImPyPy-β-C_3-NH_2 (6). Cleaved from β-Ala-PAM resin with neat 1,3-diaminopropane. UV-vis λ_{max} = 315 nm; MALDI-TOF-MS *m*/*z* 1246.68 ($C_{55}H_{65}ClN_{21}O_{10}S^+$ calculated $[M+H]^+$ 1246.46)

CtPyPyIm-(*R*)^{H_2N}γ-PyImPyPy-β-(+)-FITC (7). Cleaved from β-Ala-PAM resin with neat 3,3'-diamino-*N*-methyldipropylamine and conjugated with fluorescein-5-isothiocyanate (FITC, Invitrogen). UV-vis λ_{max} = 313, 442 nm; ESI-MS *m*/*z* 1704.3 ($C_{80}H_{83}ClN_{23}O_{15}S_2^-$ calculated $[M-H]^-$ 1704.56)

CtPyPyIm-(*R*)^{H_2N}γ-PyImPyPy-β-(+)-IPA (8). Cleaved from β-Ala-PAM resin with neat 3,3'-diamino-*N*-methyldipropylamine and conjugated with isophthalic acid. UV-vis λ_{max} = 316 nm; MALDI-TOF-MS *m*/*z* 1465.59 ($C_{67}H_{78}ClN_{22}O_{13}S^+$ calculated $[M+H]^+$ 1465.55)

The synthesis and characterization of polyamide conjugate **1** has been reported previously.[8] The synthesis and characterization of polyamide conjugates **9**-**13** are reported in Chapter 4A.

4.6.2. Quantitative DNase I footprint titrations

pGL2-VEGF-*Luc* was 5'-end-labeled and amplified as previously described.[8,9] PCR products (5'-end-labeled, 197 bp for pGL2-VEGF-*Luc*) were isolated according to standard protocols.[13] Quantitative DNase I footprint titration experiments were performed on the 5'-^{32}P-end-labeled PCR products of plasmid pGL2-VEGF-*Luc* with polyamides **2**-**8** according to standard protocols.[13] Radiolabeled DNA was equilibrated with polyamide solutions for 14-16 h at 22°C in a buffer of 10 mM Tris-HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$ at pH 7.0 prior to DNase I cleavage. Chemical sequencing reactions were performed according to published methods.[14,15] Storage phosphor autoradiography was performed on a Molecular Dynamics Typhoon 8600 phosphorimager. 18 MΩ water was obtained from an AquaMAX Ultra water purification system, and all buffers were 0.2 μm filtered.

4.6.3. Cell cultures

The human cervical cancer cell line HeLa was cultured in a 5% CO_2 atmosphere at 37°C in supplemented DMEM medium (GIBCO).[5,6] DMEM medium was supplemented with 10% fetal bovine serum (Omega Scientific) and 1% penicillin/streptomycin solution (Mediatech).

4.6.4. Confocal microscopy

Confocal microscopy experiments were performed according to published protocols.[5,6] HeLa cells were trypsinized (Mediatech) for 5 min at 37°C, centrifuged for 10 min at 4°C at 100 × g, and resuspended in fresh medium to a concentration of 1.33×10^5 cells/mL.[5,6] Incubations were performed by adding 150 μL of cells into culture dishes equipped with glass bottoms for direct imaging (MatTek). Cells were grown in the glass-bottom culture dishes for 24 h. The medium was then removed and replaced with 147 μL of fresh medium, followed by addition of 3 μL of the 100 μM solution for a final polyamide concentration of 2 μM. Cells were incubated in a 5% CO_2 atmosphere at 37°C for 12-14 h. Imaging was performed with a 40× oil-immersion objective lens on a Zeiss LSM 5 Pascal inverted laser scanning microscope. Polyamide-fluorescein conjugate fluorescence and visible light images were obtained using standard filter sets for fluorescein.[5,6]

4.6.5. Quantitative RT-PCR experiments

Quantitative RT-PCR experiments were performed using HeLa cells according to published protocols.[8,9] For cell culture experiments, HeLa cells were plated in a 24-well format with a concentration of 3×10^4 cells/mL in a volume of 500 μL. Polyamides were added to the medium at this time. Hypoxia was induced with 300 μM desferrioxamine mesylate (DFO) after 48 h. RNA was harvested after 16 h of chemical induction using an RNeasy Mini Kit (Qiagen) and reverse transcribed using PowerScript Reverse Transcriptase

(Clontech). Quantitative RT-PCR was performed using SYBR GREEN PCR Master Mix (Applied Biosystems) on an Applied Biosystems 7300 Real Time PCR System. VEGF gene expression levels were measured relative to GUSB.

4.7. Results and discussion

4.7.1. Polyamide design and synthesis

The hairpin polyamide conjugates shown in Figure 4.5 comprise two polyamide cores for match and mismatch experiments. The synthesis and activity of polyamide conjugate **1** have been previously characterized.[8-10] Polyamides **2-8** were designed to target the hypoxia response element 5'-TACGTG-3' binding site. Polyamides **2-6** were synthesized with Boc-β-Ala-PAM resin and cleaved with 1,3-diaminopropane to give the β-alanine-C_3 linker. Conjugates **2-5** were formed with a fluorescein or isophthalic acid moiety containing a thiourea or amide linkage. An unconjugated β-alanine-C_3 polyamide **6** was included in the series as a control compound. Conjugates **7** and **8** incorporate a fluorescein or isophthalic acid moiety with a positively charged β-alanine-triamine linker.

The synthesis of polyamides **9-13** has been described in Chapter 4A. These compounds bind the sequence 5'-WGGWCW-3' with affinities of $K_a > 2 \times 10^9$ M^{-1}. The nuclear localization of the fluorescein conjugate **9** is excellent across the HeLa, MCF-7, and PC3 cell lines tested, whereas the uptake of the FAM conjugate **10** is more limited but still nuclear.

4.7.2. DNase I footprint titrations

The binding affinities of polyamides **2-8** on the hypoxia response element 5'-TACGTG-3' on plasmid pGL2-VEGF-*Luc* were measured by DNase I footprint titration experiments (Figure 4.6 and Table 4.4). With the exception of polyamide **3**, all of the compounds in the series have equilibrium association constants of $K_a > 3 \times 10^9$ M^{-1}. As previously observed, the fluorescein conjugates **2** and **3** exhibit decreased binding affinities.

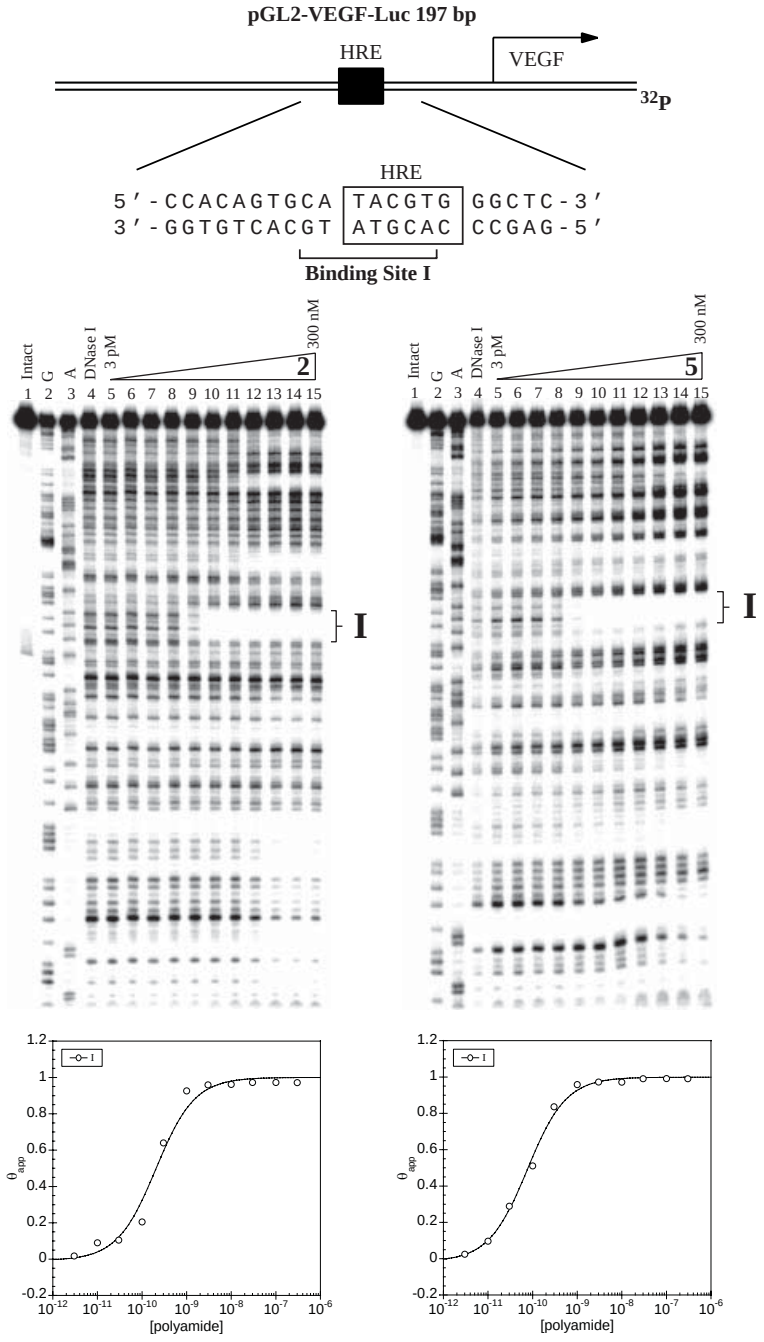

Figure 4.6. Quantitative DNase I footprint titration experiments for polyamides **2** and **5** on the 197 bp, 5'-^{32}P-end-labeled PCR product of pGL2-VEGF-*Luc*; lane 1, intact DNA; lane 2, G reaction; lane 3, A reaction; lane 4, DNase I standard; lanes 5-15, 3 pM, 10 pM, 30 pM, 100 pM, 300 pM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM polyamide, respectively. Binding isotherms are shown below each footprinting gel; θ_{norm} values were calculated according to published methods.[13]

Table 4.4. Equilibrium association constants for polyamides **2-8**[a]

Polyamide	5'-TACGTG-3'
2	3.8 (±1.2) × 10^9
3	1.0 (±0.3) × 10^8
4	1.2 (±0.2) × 10^{10}
5	1.4 (±0.1) × 10^{10}
6	1.3 (±0.2) × 10^{11}
7	3.6 (±0.9) × 10^{10}
8	2.1 (±0.6) × 10^{10}

[a] K_a (M^{-1}) values reported are the mean values from at least three DNase I footprint titration experiments; standard deviations are shown in parentheses. Assays were performed at 22°C in a buffer of 10 mM Tris-HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$ at pH 7.0.

In Chapter 4A, the difference between the affinities of the FITC and FAM conjugates was less than two-fold. In this case, FITC conjugate **2** binds the match site with K_a = 3.8 × 10^9 M^{-1}, while the FAM conjugate **3** has an affinity of 1.0 × 10^8 M^{-1}. This 38-fold difference is surprising and may be sequence-dependent.

4.7.3. Confocal microscopy

The cellular uptake of the β-alanine-C_3-linked polyamide-fluorescein conjugates **2**, **3**, and **7** was directly imaged on a confocal laser scanning microscope (Figure 4.7). HeLa cells were incubated with 2 μM polyamide for 12-14 h in a 5% CO_2 atmosphere at 37°C prior to imaging. All three polyamides showed nuclear staining that exceeds that of the medium. This result is encouraging in the case of the FAM conjugate **3** because the nuclear localization of the β-alanine-C_3-linked FAM conjugate **10** was modest, as described in Chapter 4A. The positive uptake of the β-alanine-triamine-linked fluorescein conjugate **7** suggests that this polyamide core could be more amenable than others in terms of localization.

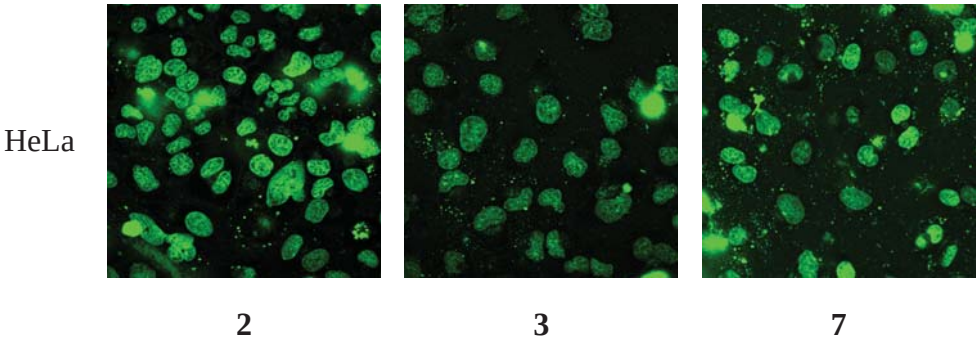

Figure 4.7. Nuclear localization of polyamides in HeLa cells after incubation at 2 μM concentration for 12-14 h at 37°C in standard culture media.

4.7.4. Quantitative RT-PCR experiments in HeLa cells

The binding of polyamide **1** to the hypoxia response element results in the downregulation of hypoxia-inducible genes, including vascular endothelial growth factor (VEGF).[8-10] HeLa cells were incubated with 0.2 μM or 1 μM polyamide for 48 h. After the incubation period, hypoxia was induced with 300 μM DFO. RNA was harvested 16 h after induction, and quantitative RT-PCR analysis was performed (Figure 4.8 and Figure 4.9).

The β-alanine-C_3-linked hairpin polyamide conjugates **2**, **4**, and **5** showed comparable levels of VEGF mRNA that were decreased relative to the untreated control. However, the lead compound **1** remained superior in downregulating the VEGF gene. The FAM conjugate **3**, which had shown good nuclear localization but decreased binding affinity, showed little activity. The high-affinity unconjugated polyamide **6** also displayed little effect, consistent with the model that polyamide conjugates exhibit improved nuclear localization. The β-alanine-triamine-linked fluorescein conjugate **7** showed moderate results, while the analogous isophthalic acid conjugate **8** was less effective. Mismatch polyamides **9-13** did not downregulate VEGF expression at the 1 μM concentration.

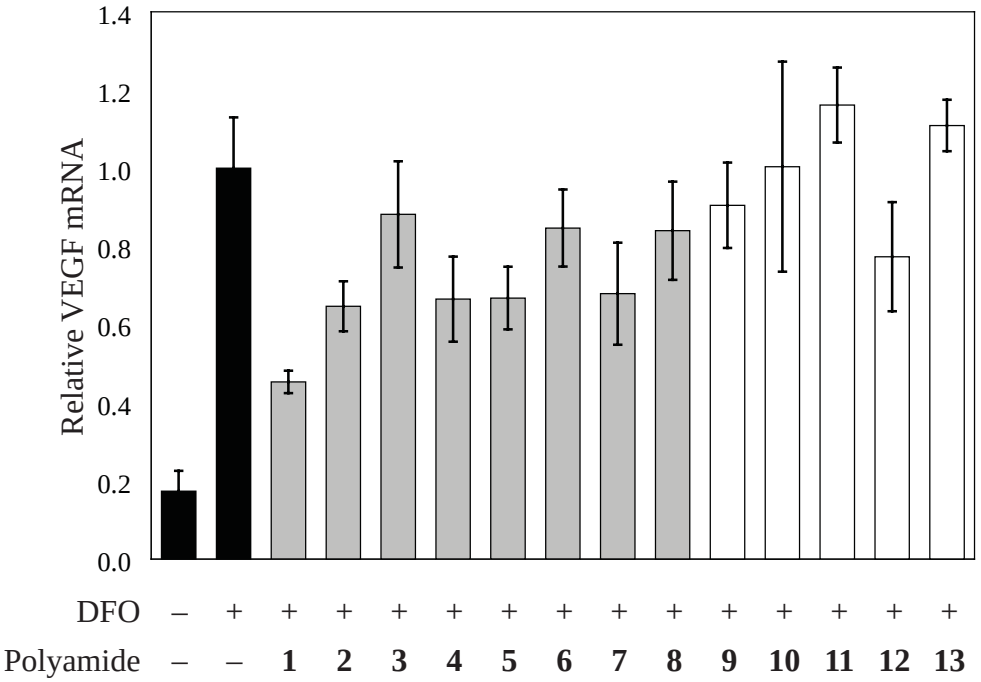

Figure 4.8. Relative levels of VEGF mRNA expression as measured by real-time quantitative RT-PCR. HeLa cells were treated with final concentration of 1 μM polyamide, and hypoxia was induced with 300 μM DFO.

Taken together, these experiments indicate that β-alanine-C_3-linked hairpin polyamide conjugates targeted to the hypoxia response element downregulate the hypoxia-inducible VEGF gene in a sequence-specific manner. Polyamide conjugates synthesized on Boc-β-Ala-PAM resin should remain viable candidates for cell culture studies. Judicious linker selection is important in determining nuclear localization.

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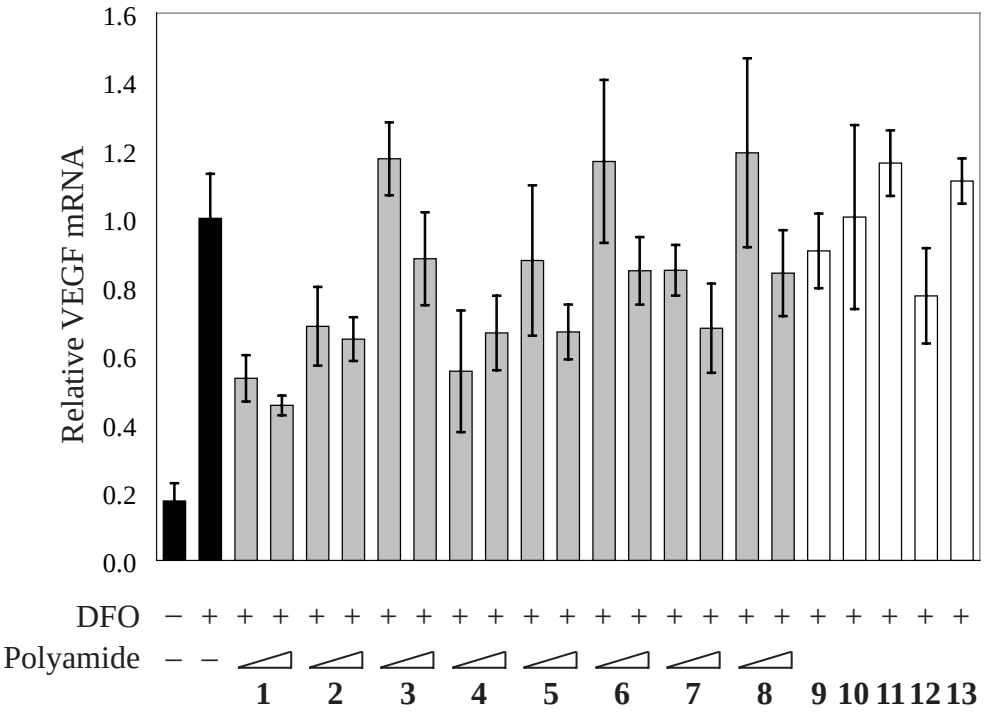

Figure 4.9. Relative levels of VEGF mRNA expression as measured by real-time quantitative RT-PCR. HeLa cells were treated with final concentration of 0.2 or 1 μM polyamide, and hypoxia was induced with 300 μM DFO.

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