Chapter 4

Cell-Permeable Protein-DNA Dimerizers

*Work on the synthesis of polyamide-peptide-FAM conjugates **13** and **16** was done in collaboration with Rachel Wang (Dervan Group; Caltech).*

Abstract

In general, polyamide-peptide-fluorescein conjugates have shown poor cell uptake. This chapter describes how such conjugates can be made to localize to the cell nucleus through subtle structural changes. Specifically, cell-permeable conjugates are described that incorporate smaller peptides with the fluorescein attached to the polyamide C-terminus. Similar peptides conjugated to fluorescein without a polyamide, exhibited primarily extracellular localization which suggests that the polyamide confers the nuclear localization to the conjugate. Polyamide-peptide-fluorescein and polyamide-peptide-isophthalic acid conjugates also retain protein-DNA dimerization activity *in vitro*. These experiments provide a foundation for performing experiments in cell culture with protein-DNA dimerizers.

4.1 Introduction to Polyamide Conjugate Cell Uptake

Several conjugates containing pyrrole-imidazole polyamides and peptides have been reported to have a variety of functions *in vitro* such as gene activation[1-3] and protein-DNA dimerization.[4-7] Experiments in cell culture have experienced difficulty partly due to the poor cellular permeability of such conjugates. Polyamides by themselves often localize to the cell nucleus[8-9] which suggests that the addition of peptides hinders uptake. The utilization of non-peptide moieties does not render them cell permeable though. For example, a polyamide conjugated to the small-molecule wrenchnolol exhibits extracellular localization.[10] Wrenchnolol[11-12] and the polyamide[9] are independently cell-permeable so it is intriguing that the resulting conjugate is not. Thus, it seems that the overall conjugate structure must govern the propensity towards nuclear uptake.

Extensive studies into the structural determinants for nuclear localization of polyamide-fluorescein conjugates have shown that seemingly minor structural variation can lead to dramatic changes in cellular localization.[9] One notable example is that the polyamide ImImPyPy-γ-ImPyPyPy-FITC localizes to the nucleus, but the compound ImImPyPy-γ-ImPyPyPy-**β**-FITC, which contains an additional **β**-alanine, is extracellular.[9] To complicate matters, changing a single Im to a Py (ImImPyPy-γ-PyPyPyPy-**β**-FITC) recovers the nuclear localization.[9] Consequently, the complex interplay of structural variables that affect cellular localization of polyamides has yielded only a weak set of guidelines.

Nevertheless, it is apparent that tiny structural changes, i.e., as simple as an Im to Py substitution, can yield conjugates with desirable nuclear localization. Accordingly, the attempts described in this chapter to discover cell-permeable protein-DNA dimerizers involved simply moving to the attachment point of the fluorescein moiety to the polyamide and using smaller peptide domains. These efforts yielded a polyamide-peptide-fluorescein conjugate that localizes to the nucleus of living HeLa, MCF-7, and PC3 cells and retains the ability to dimerize Exd to DNA. The effect of the size of the peptide moiety was probed

by synthesizing larger peptides, but it is unclear if the upper limit has been reached.

4.2 Previous Polyamide-Peptide Conjugate Uptake Studies

In order to assess the cell uptake of series of polyamide-FYPWMKG conjugates, Hans-Dieter Arndt attached fluorescein-isothiocyanate (FITC) to the N-termini of the peptide moieties via a six-carbon linker to give compounds such as **1** (Figure 4.1).[13] Laser-scanning confocal microscopy in live cells,[8-9] showed that these compounds localized primarily outside of human cancer cell lines (HeLa, SKBr4, CEM-CCL, NB4) and

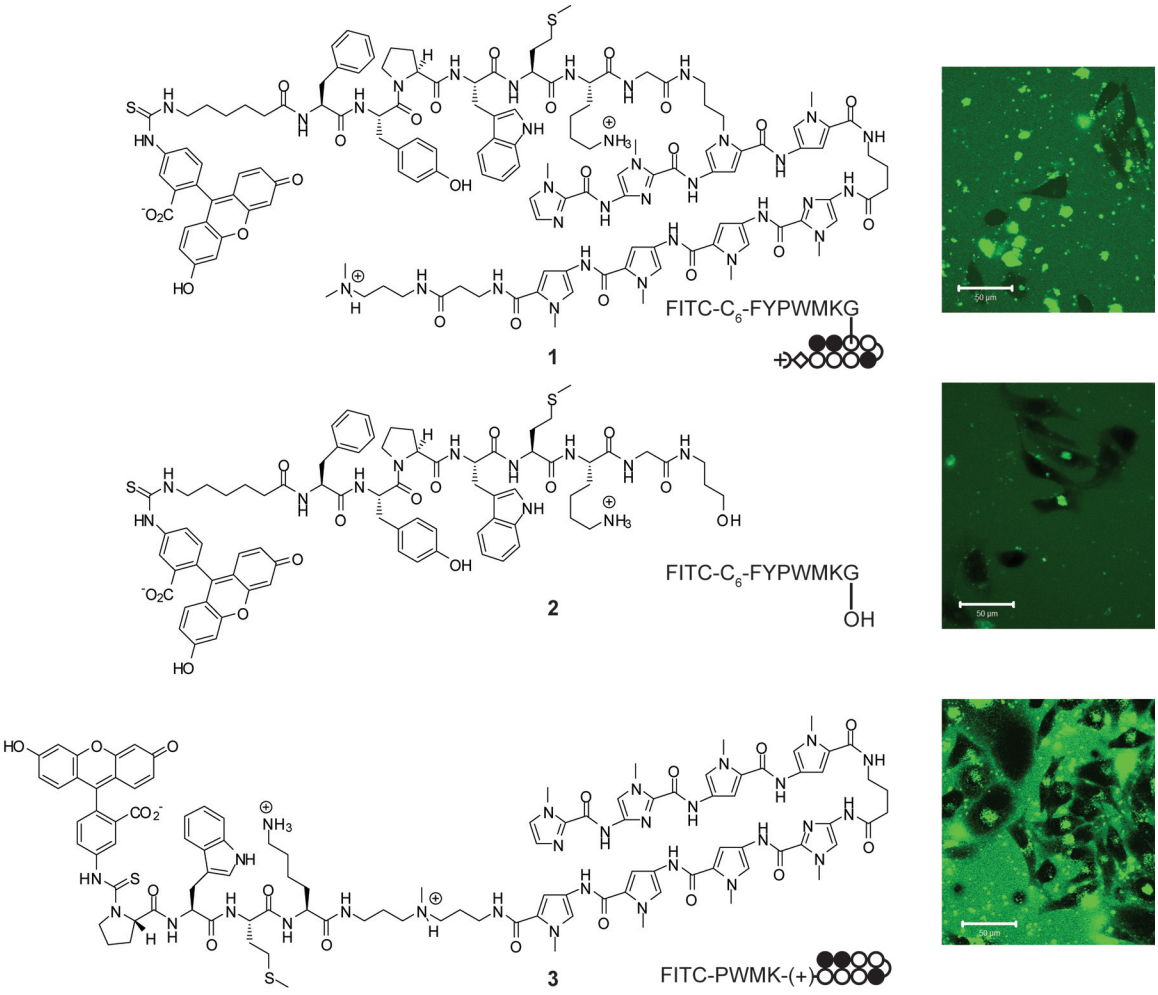


Figure 4.1 Summary of previous uptake results in HeLa cells. Polyamide-peptide-fluorescein conjugate **1** and the related peptide-fluorescein conjugate **2** show extracellular localization. Conjugate **3** is exhibits extracellular and vesicular staining. (Scale bar = 50 μm)

Drosophila cells (Kc167, S2). Similarly, the peptide-fluorescein conjugate **2** localized outside of cells. The use of the MDR pump inhibitor verapamil (100 μM), which has been shown to cause nuclear localization of some polyamides,[14] did not cause redistribution of **1** or **2**.[13] In other efforts to artificially induce cellular uptake, a pre-loaded plasmid (pDEH9) was incubated with polyamide **1** (500 nM) and a number of commercially available transfection reagents, including ExGen500 (Fermentas), Metafectene (Biontex), Escort (Sigma), and Polyfect (Qiagen), before loading the compounds onto live cells. Using the manufacturer's recommended upper and lower concentrations for the transfection reagents was not effective.[13]

Tim Best also investigated several polyamide-peptide-fluorescein conjugates, including **3**, in which similar peptides were attached to the polyamide C-terminus (Figure 4.1).[15] Unfortunately, compound **3** which contains a PWMK tetrapeptide between the polyamide and FITC does not show nuclear localization in 11 cell lines (MCF, HeLa, PC3, LN-CaP, DLD-1, 786-O, Jurkat, CEM, MEG-01, MEL, and NB4).[15] Conjugates of similar architecture bearing peptides of different sequences (DWMK and RY, with several unnatural forms of tyrosine) also yielded extracellular localization except for a couple of instances in CEM cells with the RY-containing compounds.[15]

Some control experiments with compounds **4**-**6** performed by Hans-Dieter Arndt,[13] suggested that attachment of the FITC directly to the polyamide C-terminus might be more favorable for cell uptake (Figure 4.2). Most notably, conjugate **6** which contains a tryptamine conjugated to an internal pyrrole yielded a modest degree of nuclear localization.[10] The structural difference is small between the appendage off the internal pyrrole of **6** and the WM conjugates described in Chapter 2. Thus, a related polyamide-WM conjugate was investigated, replacing the tryptamine and eight-carbon linker of **6** with a WM dipeptide and a six-carbon linker.

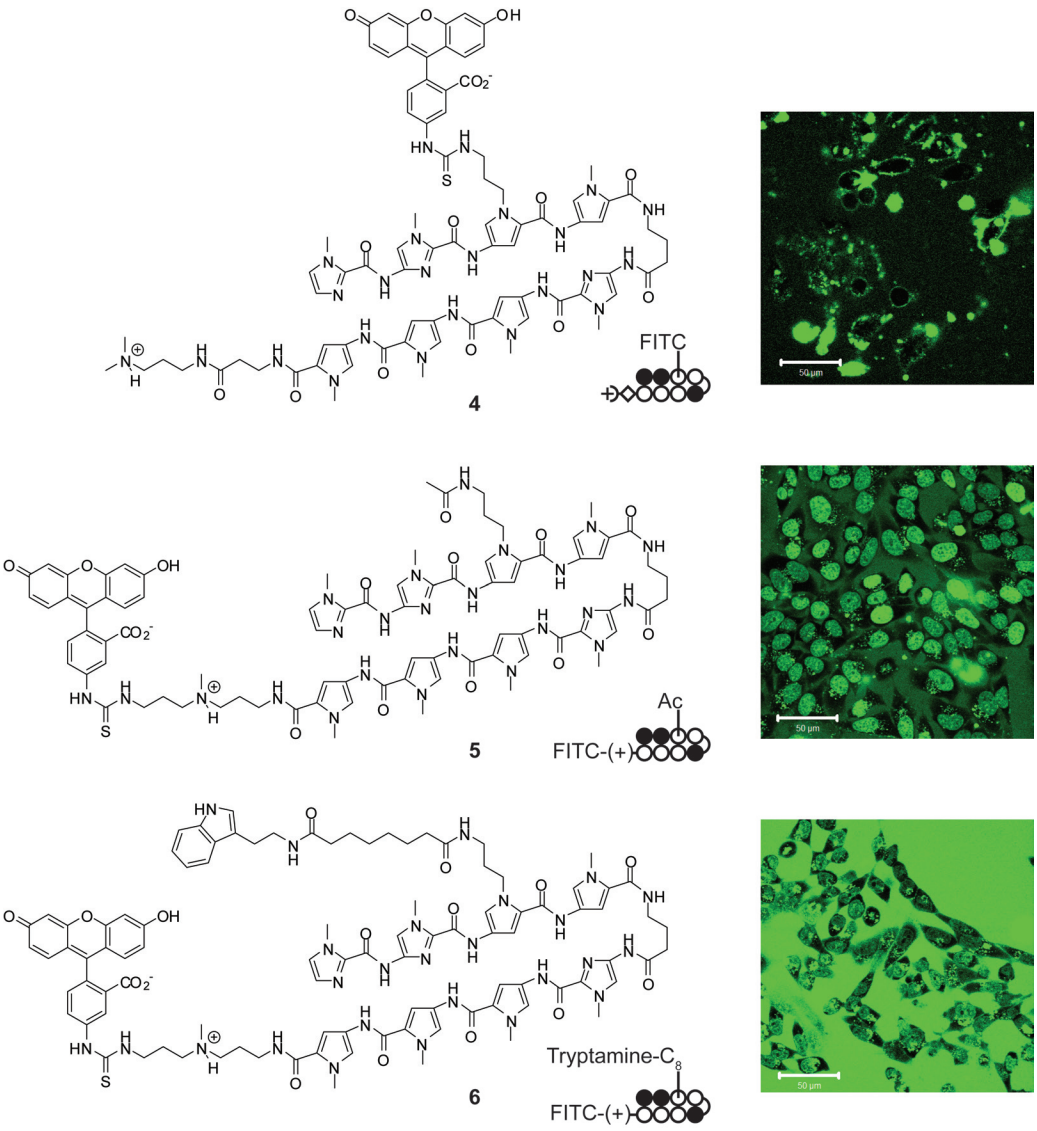


Figure 4.2 Summary of more previous uptake results in HeLa cells. Branched polyamide-FITC conjugate **4** shows extracellular and membranous staining. Polyamide-FITC conjugates **5** and **6** both show partial nuclear staining. (Scale bar = 50 μm)

4.3 Discovery of Cell-Permeable Protein-DNA Dimerizers

To synthesize the desired conjugates, resin-bound ImImPyPy-γ-ImPyPyPy-polyamide **7** was cleaved by treating with the mono-BOC protected diamine **8** (Figure 4.3). The resulting C-terminally BOC-protected polyamide **9** was coupled to the desired peptide, deprotected with TFA, and conjugated to fluorescein via an amide bond (FAM). This strategy was used to synthesize of a series of polyamide-peptide-FAM conjugates **10**-**16** for cell uptake studies (Figures 4.4 to 4.5). The natural WM conjugate **10** yielded a strong

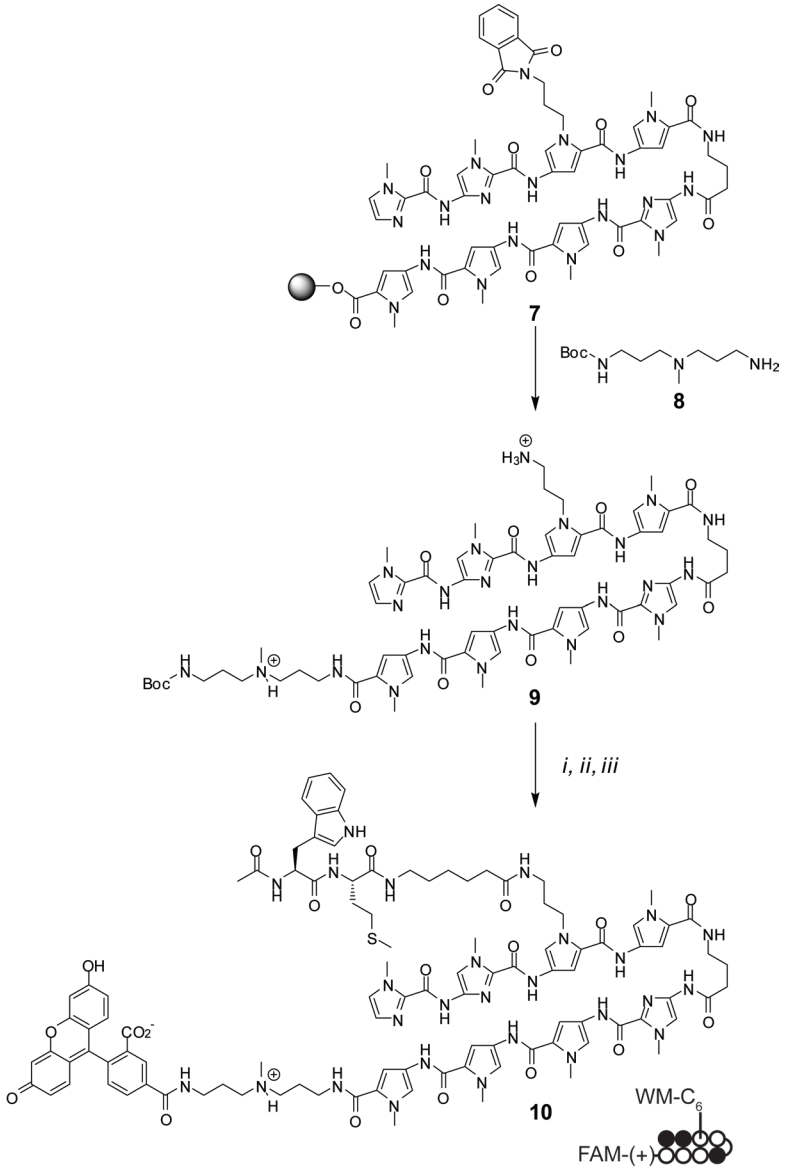


Figure 4.3 Synthesis of polyamide-peptide-fluorescein conjugates: *i*) **17**, HBTU, 0.1 M DIEA in DMF, *ii*) 50% TFA/DCM, and *iii*) 5-carboxyfluorescein succinimidyl ester.

nuclear fluorescence stain in HeLa, MCF-7 and PC3 cells (Figure 4.4). The AM conjugate **11** also showed nuclear fluorescence albeit with higher levels in the media, suggesting the tryptophan may positively impact nuclear localization. Unnatural peptide conjugates **12** (D-Trp-L-Met) and **13** (D-Trp-D-Met) yielded similarly strong nuclear localization to **10** (L-Trp-L-Met) suggesting that the stereochemistry of the tryptophan and methionine can be altered. This may be useful for future biological studies given that previous work has

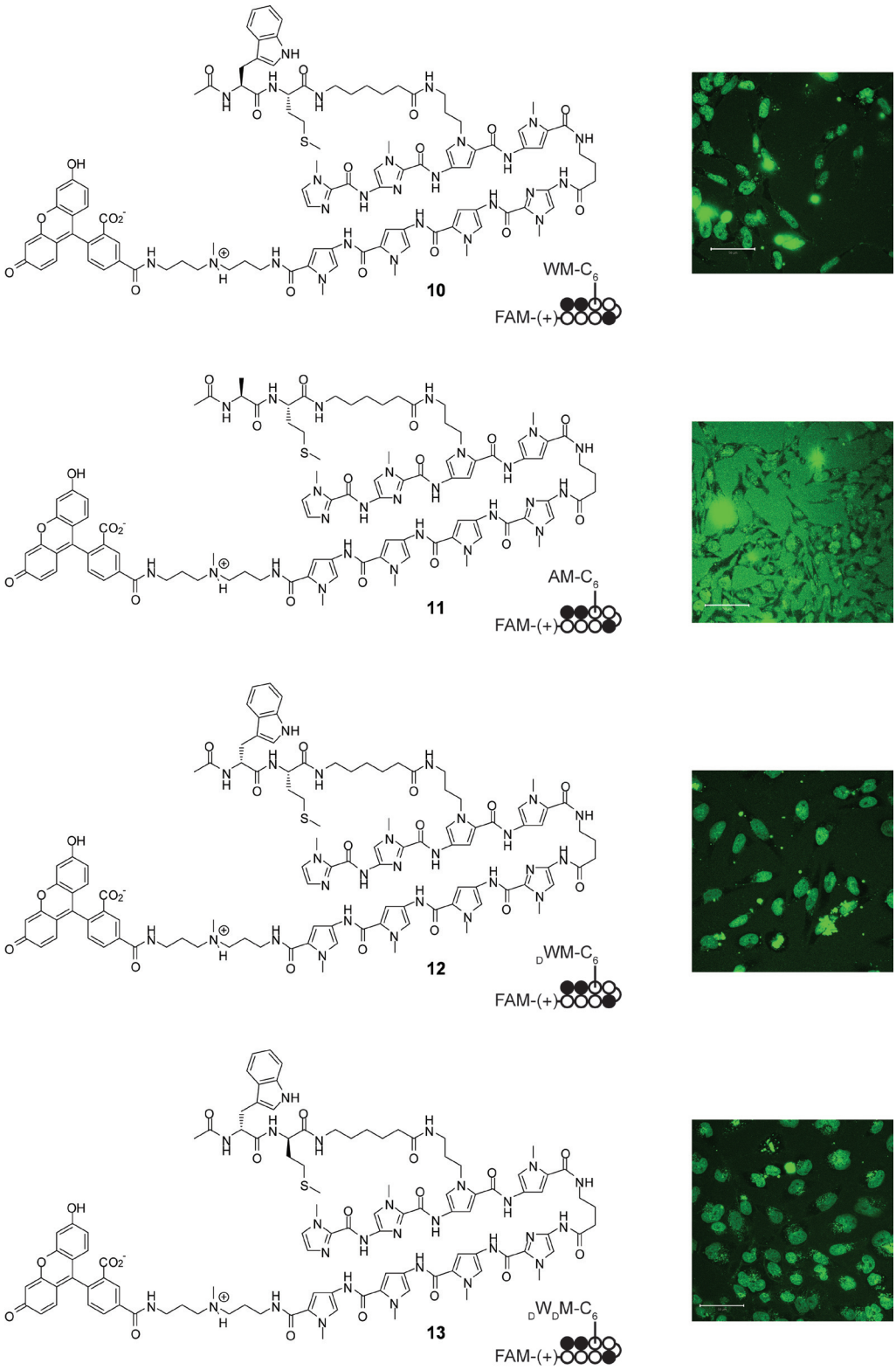


Figure 4.4 Uptake results in HeLa cells for polyamide-dipeptide-fluorescein conjugates. All compounds **10**-**13** show primarily nuclear localization, but the Trp to Ala conversion (**10** to **11**) decreases the apparent nuclear fluorescent intensity relative to the media. (Scale bar = 50 μm)

shown D-Trp-L-Met conjugates can dimerize Exd at higher temperatures (up to 37 °C) than natural dipeptides[7] and unnatural peptides should be resistant to proteolysis by endogenous proteases.

Analogous polyamide-YPWM tetrapeptide conjugates (**14**-**16**) were investigated to probe the upper size limit for the peptide (Figure 4.5). The YPWM conjugate **14** yielded strong nuclear fluorescence with some vesicular and membranous staining also apparent. Similar to the results with the AM conjugate **11**, the YPAM conjugate **15** showed higher levels of fluorescence in the media than YPWM conjugate **14**. Complete conversion of the amino acids of the YPWM peptide to the unnatural D-form (**16**) had a markedly negative impact on nuclear localization, with only modest levels of nuclear fluorescence visible. The apparent difference in uptake of **14** and **16** can be explained at least two different ways. First, it may be possible that the peptide of **14**, but not **16**, is degraded by proteases to generate smaller metabolites which render it cell permeable. Alternatively, the peptide may directly interact with some stereoselective protein involved in active uptake.[16]

To determine the uptake of peptide-fluorescein conjugates without polyamides **18** and **20** were investigated (Figure 4.6). The conjugates were synthesized by coupling **17** or **19** to mono-BOC protected diamine **8**, deprotected with TFA, and conjugated to FAM. Both peptide-FAM conjugates **18** and **20** exhibit primarily extracellular fluorescence (Figure 4.7). This suggests that the polyamide of **10** and **14** is responsible for trafficking the conjugate to the nucleus.

4.4 Dimerization of Exd with Cell-Permeable Conjugates

Electrophoretic mobility shift assays were then performed to determine the ability of cell-permeable conjugates to dimerize Exd. Polyamide-isophthalic acid (IPA) conjugates have been recently reported to possess similar cell permeability to their corresponding fluorescein conjugates.[17] Thus, a non-fluorescent IPA-polyamide-peptide conjugate **21** as well a control IPA conjugate without a peptide **22** were also investigated (Figure 4.8).

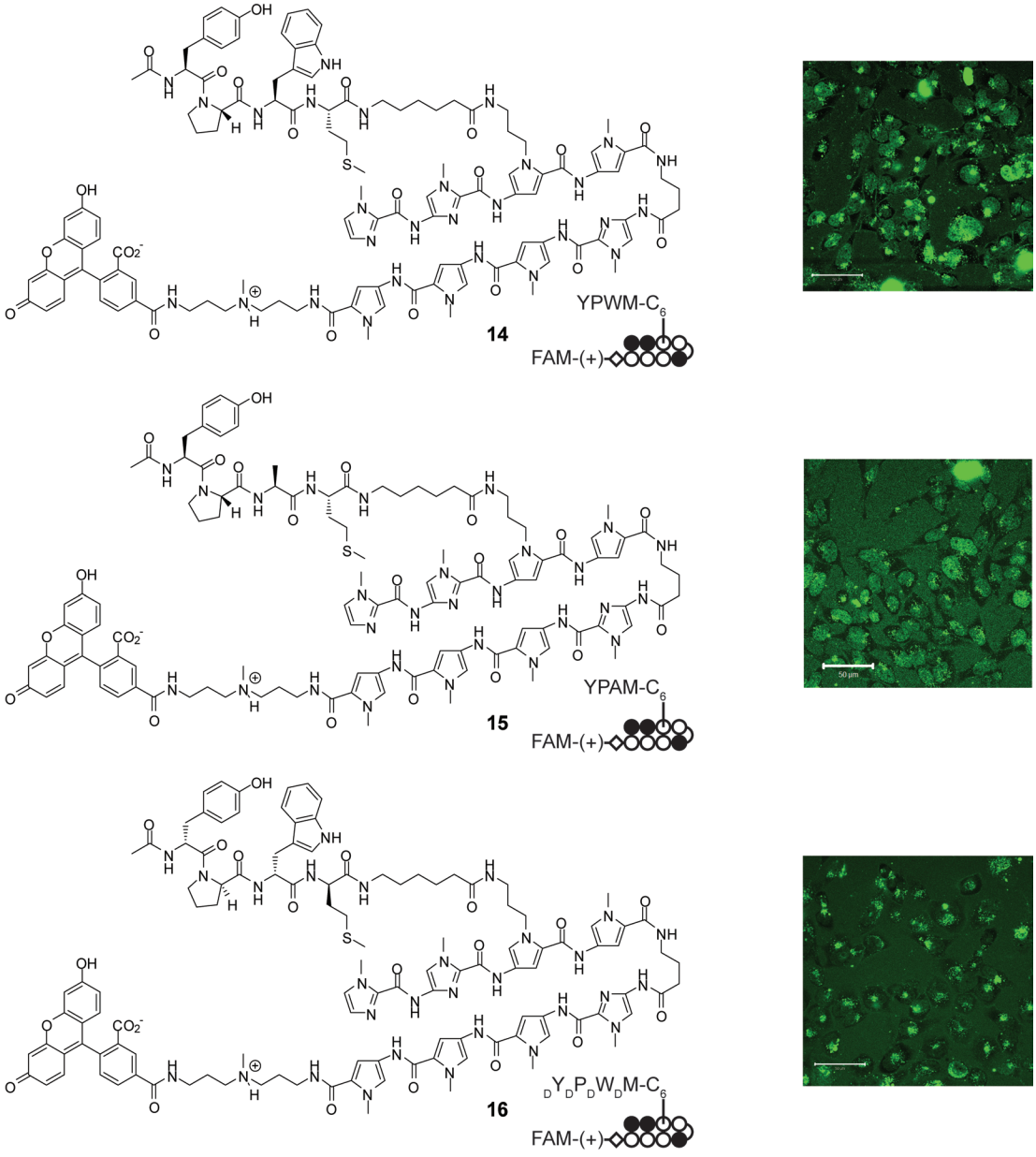


Figure 4.5 Uptake results in HeLa cells for polyamide-tetrapeptide-fluorescein conjugates. The natural YPWM conjugate **14** shows nuclear, membranous, vesicular and extracellular localization. The YPAM conjugate **15** shows weaker nuclear and extracellular staining. The unnatural YPWM conjugate **16** shows a strong vesicular and a weak nuclear staining pattern. (Scale bar = 50 μm)

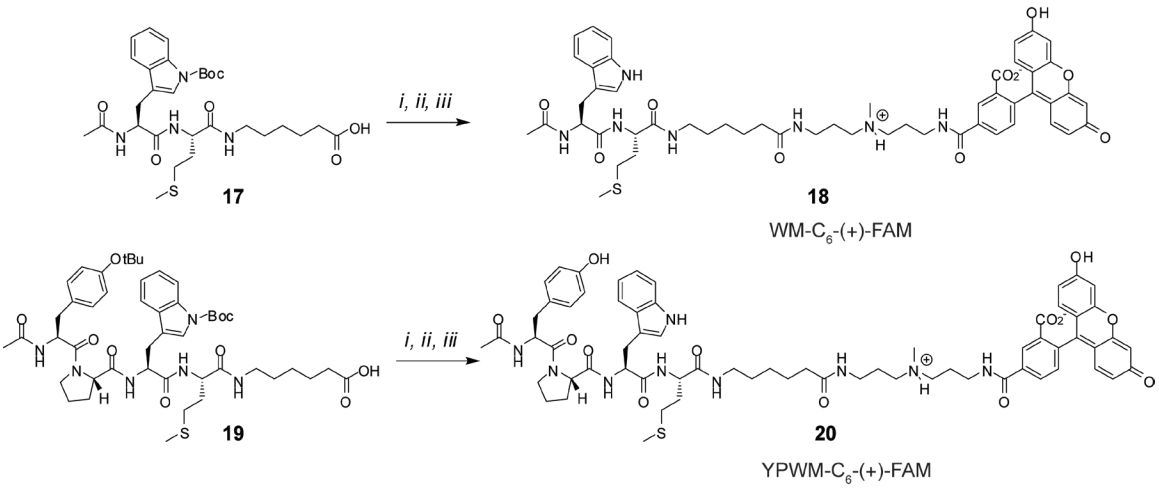
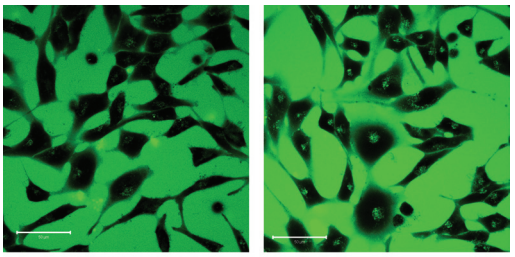


Figure 4.6 Synthesis of YPWM and WM peptide-fluorescein conjugates. *i*) **17** or **19**, HBTU, 0.1 M DIEA in DMF, *ii*) 50% TFA/DCM, and *iii*) 5-carboxyfluorescein succinimidyl ester.



WM-C_6-(+)-FAM **18**

YPWM-C_6-(+)-FAM **20**

Figure 4.7 Uptake results in HeLa cells for peptide-fluorescein conjugates. Both the WM (**18**) and YPWM (**20**) conjugates show primarily extracellular staining. (Scale bar = 50 μm)

Polyamide-WM-FAM conjugate **10** yielded a significantly higher gel shift than AM conjugate **11** or Exd alone at 4 °C (Figure 4.9). Although Exd's binding affinity in the presence of **10** (K_a = 1 ± 2 × 10^9 M^{-1}) was comparable to that of the similar conjugate lacking the FAM (K_a = 7 ± 2 × 10^8 M^{-1}, Chapter 2)[7], the fraction of DNA shifted by **10** was significantly lower (Θ_{app} = 0.54 ± 0.02 compared to 0.84 ± 0.06, Chapter 2)[7] even at this lower temperature. At a higher temperature (20 °C) polyamide-WM-FAM and -IPA conjugates with the unnatural D-Trp (**12** and **21**) yielded lower binding affinities (K_a's = 4 ± 2 and 2 ± 1 × 10^8 M^{-1}) but with higher fractions of complex formation (0.74 ± 0.08 and 0.74 ± 0.04).

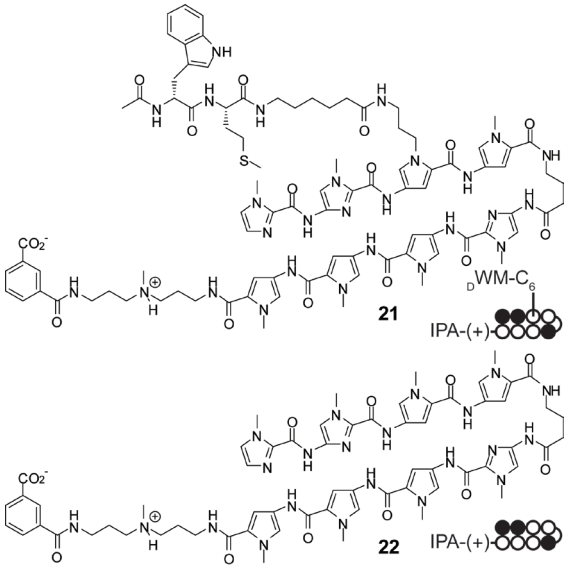


Figure 4.8 Structures of polyamide-peptide-isophthalic acid (IPA) conjugates.

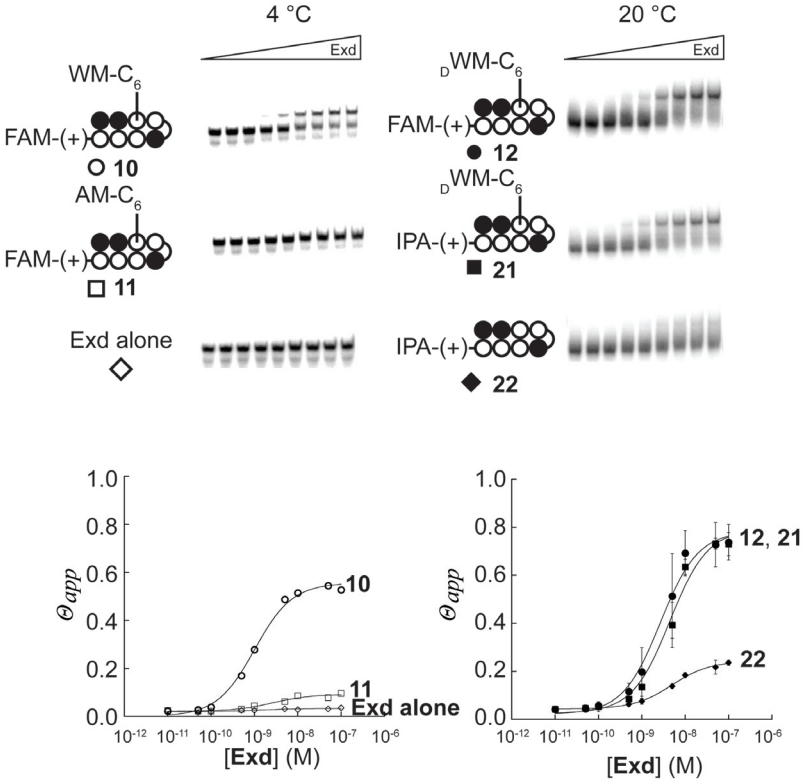


Figure 4.9 Gel shift experiments with conjugates. Polyamide-WM-FAM conjugate **10** shows a significant complex formation with Exd at 4 °C when compared to the AM control compound **11** or Exd alone. Polyamide-WM-FAM (**12**) and -IPA (**21**) conjugates which contain an unnatural D-Trp show significant complex formation with Exd at 20 °C compared to the no peptide IPA control (**22**). Conjugates were added at a concentration of 50 nM and Exd was titrated from 10 pM to 100 nM.

4.5 Conclusions

Intriguingly, the stereochemistry of the dipeptide conjugates did not appear to affect their nuclear localization, but the unnatural tetrapeptide conjugate exhibited substantially reduced nuclear staining compared to the natural enantiomer. It is unclear what causes the tetrapeptide conjugates to yield such apparent differences in cell uptake, but it is possible that the natural peptides are unstable in cell culture. Nonetheless, the presumably stable unnatural dipeptide conjugates localize to the nucleus and dimerize Exd. Thus, cell-permeable polyamide-peptide conjugates have been created that retain the ability to facilitate Exd-DNA dimerization *in vitro*. These compounds provide the foundation for cell culture and fruit fly experiments in an ongoing collaboration with the Ansari Lab (University of Madison, Wisconsin).

4.6 Experimental Details

General Synthetic Procedures. Polyamide monomers were synthesized by previously reported procedures.[18,19] Polyamides were synthesized on either PAM,[18] oxime,[20] or Marshall-Liener resin (Appendix D). All peptides were synthesized using Fmoc-based protocols on SASRIN (Bachem) or Wang resin (Novabiochem).[21] All polyamides were purified by preparative HPLC with either a Waters C18 Delta-Pak (15 μm, 25 × 100 mm, 300 Å), Varian Dynamax Microsorb C8 (250 × 21.4 mm, 300 Å), or Phenomenex C18 Gemini (5 μm, 250 × 21.2 mm, 110 Å) ramping from 10% A buffer (0.1% TFA in water) with acetonitrile as the B buffer. In general, the best purification was obtained with the Gemini column. Analytical High-Pressure Liquid Chromatography (HPLC) was performed with a Beckman Gold system equipped with a diode array 168 Detector and a 125 Solvent module using a Phenomenex Gemini C_{18} column (5 μm particle size, 250 × 4.60 mm). All conjugates were ≥95% pure before cell uptake and gel shift experiments were performed.

***N*-Boc-3,3′-diamino-*N*′-methyldipropylamine (8)**. This compound was prepared as

described previously.[22]

ImImPy$^{(propylamine)}$Py-γ-ImPyPyPy-(+)-BOC (9). This polyamide was synthesized on oxime resin according to standard procedures.[20] 255.1 mg resin cleaved with **8** (~300 μL) in 1 mL DMF at 50 °C for 13.5 h. Total yield, 7.66 μmol of a white powder (9.2% yield). (MALDI-TOF MS) $[M + H]^+$ calcd for $C_{63}H_{84}N_{23}O_{11}^+$ 1338.7, observed 1338.7.

ImImPy$^{(Trp\text{-}Met\text{-}\varepsilon Ahx\text{-}propylamine)}$Py-γ-ImPyPyPy-(+)-FAM (10). The peptide Ac-Trp(Boc)-Met-εAhx-OH (2.0 mg, 3.4 μmol) was dissolved in 200 μL 0.1 M DIEA in DMF and activated at 20 °C for ~5 min with 200 μL of a solution of PyBOP (total 5.4 mg, 10.5 μmol). The polyamide **9** (1.47 μmol) was dissolved in 200 μL of 0.1 M DIEA in DMF and added to the activated peptide. The resulting polyamide-peptide conjugate intermediate was precipitated with diethyl ether (10 mL) cooled on dry ice and centrifuged for 10 min at 20 °C. The supernatant was decanted and the resulting pellet was dried briefly (<30 sec) with $N_{2(g)}$. The Boc moiety was removed by treating with 50% TFA/DCM and incubating at 20 °C for 25 min. The deprotected conjugate was precipitated with diethyl ether, collected by centrifugation, and dried as before. The pellet was dissolved in 800 μL 1.0 M DIEA in DMF and 3 μmol of 5-carboxyfluorescein succinimidyl ester (Molecular Probes) was added as a 0.1 M solution in DMSO. The resulting compound was purified by preparative HPLC and lyophilized to yield 364 nmol a yellow-orange solid (~25% yield). (MALDI-TOF MS) $[M + H]^+$ calcd for $C_{103}H_{118}N_{27}O_{19}S^+$ 2068.9, observed 2068.9.

ImImPy$^{(Ala\text{-}Met\text{-}\varepsilon Ahx\text{-}propylamine)}$Py-γ-ImPyPyPy-(+)-FAM (11). The conjugate was synthesized in a similar manner to conjugate **10** using the peptide Ac-Ala-Met-εAhx-OH (2.1 mg, 5.6 μmol) activated with PyBOP (5.4 mg, 10.4 μmol) coupled to polyamide **9** (1.47 μmol). The resulting intermediate was then coupled to 5-carboxyfluorescein succinimidyl ester (3.0 μmol). The final product was purified by preparative HPLC and lyophilized to yield 671 nmol of a yellow-orange solid (~46% yield). (MALDI-TOF MS) $[M + H]^+$ calcd for $C_{95}H_{113}N_{26}O_{19}^+$ 1953.8, observed 1953.9.

ImImPy$^{(\omega\text{-}Trp\text{-}Met\text{-}\varepsilon Ahx\text{-}propylamine)}$Py-γ-ImPyPyPy-(+)-FAM (12). The conjugate was

synthesized in a similar manner to conjugate **10** using the peptide Ac-D-Trp-Met-εAhx-OH (~1.9 μmol) activated with PyBOP (6 μmol) coupled to polyamide **9** (1.0 μmol). The resulting intermediate was then coupled to 5-carboxyfluorescein succinimidyl ester (1.5 μmol). The final product was purified by preparative HPLC and lyophilized to yield 411 nmol of a yellow-orange solid (~41% yield). (MALDI-TOF MS) $[M + H]^+$ calcd for $C_{103}H_{118}N_{27}O_{19}S^+$ 2068.9, observed 2069.0.

ImImPy$^{(D\text{-}Trp\text{-}D\text{-}Met\text{-}\varepsilon Ahx\text{-}propylamine)}$Py-γ-ImPyPyPy-(+)-FAM (13). The conjugate was synthesized in a similar manner to conjugate **10** using the peptide Ac-D-Trp-D-Met-εAhx-OH (2.3 mg, 3.9 μmol) activated with PyBOP (5.4 mg) coupled to polyamide **9** (1.35 μmol). The resulting intermediate was then coupled to 5-carboxyfluorescein succinimidyl ester (1.5 μmol). The final product was purified by preparative HPLC and lyophilized to yield 224 nmol of a yellow-orange solid (~17% yield). (MALDI-TOF MS) $[M + H]^+$ calcd for $C_{103}H_{118}N_{27}O_{19}S^+$ 2068.9, observed 2068.8.

ImImPy$^{(Tyr\text{-}Pro\text{-}Trp\text{-}Met\text{-}\varepsilon Ahx\text{-}propylamine)}$Py-γ-ImPyPyPy-(+)-FAM (14). The conjugate was synthesized in a similar manner to conjugate **10** using the peptide Ac-Tyr(OtBu)--Pro-Trp(BOC)-Met-εAhx-OH (~3 μmol) activated with PyBOP (~6 μmol) coupled to polyamide **9** (1.7 μmol). The resulting intermediate was then coupled to 5-carboxyfluorescein succinimidyl ester (3 μmol). The final product was purified by preparative HPLC and lyophilized to yield 448 nmol of a yellow-orange solid (~26% yield). (MALDI-TOF MS) $[M + H]^+$ calcd for $C_{117}H_{134}N_{29}O_{22}S^+$ 2329.0, observed 2329.0.

ImImPy$^{(Tyr\text{-}Pro\text{-}Ala\text{-}Met\text{-}\varepsilon Ahx\text{-}propylamine)}$Py-γ-ImPyPyPy-(+)-FAM (15). The conjugate was synthesized in a similar manner to conjugate **10** using the peptide Ac-Tyr(OtBu)-Pro-Ala-Met-εAhx-OH (~3 μmol) activated with PyBOP (~6 μmol) coupled to polyamide **9** (1.7 μmol). The resulting intermediate was then coupled to 5-carboxyfluorescein succinimidyl ester (3 μmol). The final product was purified 2x by preparative HPLC and lyophilized to yield 68 nmol of a yellow-orange solid (~4% yield). (MALDI-TOF MS) $[M + H]^+$ calcd for $C_{109}H_{129}N_{28}O_{22}^+$ 2214.0, observed 2214.1.

ImImPy$^{(D\text{-}Tyr\text{-}D\text{-}Pro\text{-}D\text{-}Trp\text{-}D\text{-}Met\text{-}\varepsilon Ahx\text{-}propylamine)}$Py-γ-ImPyPyPy-(+)-FAM (16). The conjugate was synthesized in a similar manner to conjugate **10** using the peptide Ac-D-Tyr(OtBu)-D-Pro-D-Trp(Boc)-D-Met-εAhx-OH (6 mg, ~6.6 μmol) activated with PyBOP (5.4 mg) coupled to polyamide **9** (1.32 μmol). The resulting intermediate was then coupled to 5-carboxyfluorescein succinimidyl ester (4 μmol). The final product was purified by preparative HPLC and lyophilized to yield 224 nmol of a yellow-orange solid (~17% yield). (MALDI-TOF MS) $[M + H]^+$ calcd for $C_{117}H_{134}N_{29}O_{22}S^+$ 2329.0, observed 2328.8.

Trp(Boc)-Met-εAhx-OH (17). The peptide was synthesized by Fmoc methods[21] using SASRIN resin and cleaved with 2.5 mL of cleavage mixture (89:5:5:1 DCM:EDT:TES:TFA) 4x for 15 min each. Each 2.5 mL cleavage mixture was cooled on an ice bath and neutralized by the addition of 50 μL pyridine. The peptide was partitioned between 100 mL of 1M $KHSO_4$ and 100 mL EtOAc. The organic layer was washed 1x with 100 mL of brine, dried over anhydrous sulfate, filtered and evaporated under reduced pressure to give a yellow film. Added a minimal amount (<1 mL) of MeOH to dissolve the film which then added to ~45 mL of ice cold water to precipitate the peptide which was frozen and lyophilized to give a white powder (estimated 80% purity by analytical HPLC). (ESI-MS) $[M-H]^-$ calcd for $C_{29}H_{41}N_4O_7S^-$ 589.3, observed 589.1.

Trp-Met-εAhx-(+)-FAM (18). Peptide **17** (2.2 mg, 3.7 μmol) was activated with PyBOP (10.3 mg, 19.8 μmol) in 0.1 M DIEA in DMF (200 μL) for ~5 min at RT before adding to amine **8** (6 μmol). After ~1 hour, the solution was added to 3 mL of saturated NH_4Cl extracted 3x with 3 mL of DCM. The combined organic layer was dried with anhydrous sodium sulfate, filtered, and dried under reduced pressure to yield an oil. The Boc group was removed with 2 mL of 50% TFA/DCM for 25 min at RT and dried under reduced pressure. Benzene (~2 mL) was added to azeotrope the solvent under reduced pressure to give a crude film which was dissolved in 2 mL of 1 M DIEA in DMF. The fluorescent moiety 5-carboxyfluorescein succinimidyl ester (3.7 μmol as a 0.05 M solution in DMSO) was added and the resulting crude product was purified by preparative HPLC to give 1.42

μmol of a yellow-orange solid (~38% yield). (ESI-MS) $[M+H]^+$ calcd for $C_{52}H_{62}N_7O_{10}S^+$ 976.4, observed 976.2.

Tyr(OtBu)-Pro-Trp(Boc)-Met-εAhx-OH (19). The peptide was synthesized by Fmoc methods[21] using SASRIN resin and cleaved by treating the resin 4x with the cleavage mixture (1:1:98 TFA:EDT:DCM) draining into a flask cooled on ice. 50 μL of pyridine was added to neutralize the solution after each cycle. The resin was rinsed with 3-5 mL of DCM (2x) and MeOH (2x) twice and the solvent was removed under reduced pressure, dissolved in MeOH (~8 mL), and purified by preparative HPLC. Fractions with the peptide were pooled and lyophilized to give a white powder. (ESI-MS) $[M-H]^-$ calcd for $C_{47}H_{66}N_6O_{10}S^-$ 905.5, observed 905.4.

Tyr-Pro-Trp-Met-(+)-FAM (20). Peptide **19** (3.3 mg, 3.65 μmol) was activated with PyBOP, deprotected, and coupled to fluorescein as described for conjugate **18** to yield 1.4 μmol of a yellow-orange solid (~38% yield). (ESI-MS) $[M+H]^+$ calcd for $C_{66}H_{78}N_9O_{13}S^+$ 1236.5, observed 1236.4.

ImImPy$^{(D\text{-Trp-Met--}\varepsilon\text{Ahx-propylamine})}$Py-γ-ImPyPyPy-(+)-IPA (21). Based on previously published procedures,[17] the conjugate was synthesized in a similar manner to conjugate **10** using the peptide Ac-D-Trp-Met-εAhx-OH (~3.4 μmol) activated with PyBOP (6 μmol) coupled to polyamide **9** (1.0 μmol). The resulting intermediate was then coupled to excess isophthalic acid (IPA, 20 μmol) pre-activated (~1-2 min) with PyBOP (40 μmol) in 600 μL 1 M DIEA in DMF. The final product was purified by preparative HPLC and lyophilized to yield 504 nmol of a white solid (~50% yield). (MALDI-TOF MS) $[M + H]^+$ calcd for $C_{90}H_{112}N_{27}O_{16}S^+$ 1858.9, observed 1858.8.

ImImPyPy-γ-ImPyPyPy-(+)-IPA (22). Based on previously published procedures,[17] the parent polyamide ImImPyPy-γ-ImPyPyPy-(+)-NH_2 (~1.1 μmol) was coupled to excess isophthalic acid (IPA, 20 μmol) pre-activated (~1-2 min) with PyBOP (40 μmol) in 600 μL 1 M DIEA in DMF and purified by preparive HPLC. The final product was lyophilized to yield 414 nmol of a white solid (~37.6% yield). (MALDI-TOF MS) $[M + H]^+$ calcd for

$C_{64}H_{75}N_{22}O_{12}S^+$ 1343.6, observed 1343.4.

Cell culture and confocal microscopy experiments. All cell culture and confocal microscopy was performed essentially as described previously.[9] Briefly, MCF-7 and PC3 cells were cultured in 10% FBS, 1% penicillin/streptomycin supplemented RPMI 1640 at 37 °C in an incubator with 5% CO_2 according to ATCC recommended procedures. HeLa cells were cultured in similarly supplemented DMEM under the same conditions according to ATCC recommended procedures. Before confocal microscopy, cells were trypsinized for 5 min at 37 °C, centrifuged for 5 min at 900 g, and resuspended to a concentration of 3.33×10^5 cells/mL, and 150 μL was plated on glass bottom culture plates (MatTek) and allowed to adhere for 24 hours. The medium was removed and replaced with fresh medium supplemented with the desired conjugate (150 μL final media volume with 2 μM final conjugate concentration). Cells were incubated as described above for 10-14 hours before imaging on a Zeiss LSM 5 Pascal inverted laser scanning micrscope. The optical slice was set to 2.2 μm (pinhole size = 181) and images were line averaged 4 or 8 times using a 40× oil immersion lens (plan-neofluor) using standard fluorescein filters. Stock solutions of conjugates usually contained small amounts of precipitate that was apparent upon centrifugation. Higher percentages of DMSO led to complete solubilization, but the localization results remained the same regardless. Thus, stock solutions were sonicated and vortexed immediately prior to use and the lowest possible final DMSO concentration in the media was employed at ≤0.2% for all samples (usually much lower).

Gel shift experiments. Gel shift experiments shown in Figure 4.9 were performed according to previously described procedures at 4 or 20 °C (Chapter 2).[7]

4.7 References

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