Chapter 5

Toward a Synthetic HIF-1α Mimic

This project was done in collaboration with Nick Nickols and Mike Brochu (Dervan Group; Caltech).

*The synthesis of polyamides **11**, **20** and all RT-PCR experiments were done by Nick Nickols (Dervan Group; Caltech).*

Abstract

The ability to up-regulate any desired gene through the rational design cell-permeable small molecules would be an incredibly useful tool and potentially have therapeutic value. This chapter describes efforts towards this goal that builds on the Dervan group's previous success at targeting the hypoxia response element (HRE) in the promoter region of vascular endothelial growth factor (VEGF). In living cells, hypoxia inducible factor (HIF-1α) binds to the VEGF HRE and recruits a co-activator, CREB binding protein (CBP), which activates gene expression. To mimic HIF-1α, polyamides that bind to the VEGF HRE have been conjugated to small molecules that bind to CBP. Several small-molecule-polyamide conjugates with different shapes (branched and linear) have been synthesized and their DNA-binding and cell-permeability properties have been determined. Each compound has also been assessed for its ability to affect VEGF gene expression in cell culture experiments. Remarkably, the small molecules without the polyamide activated gene expression by themselves.

5.1 Introduction

The ability to up-regulate the expression of any given gene using small molecules would be a powerful tool for molecular biology.[1-4] Cells up-regulate gene expression by using modular transcription factors that possess a sequence specific DNA binding domain and an activation domain that is capable recruiting the RNA polymerase II complex to the gene promoter.[5] RNA polymerase II subsequently transcribes the coding region into mRNA.[6-7] Promoter regions are loosely defined as -3000 to +300 base pairs from the start site of a given gene.[8] Activation domains of transcription factors can recruit the RNA polymerase complex through *direct* physical association or *indirectly* through co-activator proteins which mediate the interaction.[9-10] This chapter describes efforts toward the development of synthetic transcription factor mimics that are designed to recruit a co-activator to the promoter region of vascular endothelial growth factor (VEGF) in a living cell.

In the presence of low oxygen VEGF is activated by the hypoxia-inducible factor 1-α (HIF-1α)/aryl hydrocarbon receptor (ARNT) heterodimer transcription factor complex that binds the hypoxia response element (HRE) in the VEGF promoter.[11-12] Under hypoxic conditions HIF1-α becomes hydroxylated and is transported from the cytoplasm to the nucleus where it binds to the HRE of VEGF.[13] HIF-1α activates VEGF indirectly using the co-activator CREB binding protein (CBP) to mediate the recruitment of the RNA polymerase II complex.[14] CBP is a general co-activator known to mediate the interaction between numerous transcription factors and RNA polymerase II.[15] Several small molecules have been reported to bind to CBP or the related co-activator p300 (Figure 5.1).[16-20] Furthermore, at least two small molecules have been reported to bind specifically to the HRE of VEGF, including the polyamide CtPyPyIm-γ-PyImPyPy-(+)-FITC.[21-22] The design of small-molecule mimics of HIF-1α incorporated both CBP and HRE-binding small molecules into conjugates intended to recruit CBP to the HRE of VEGF to activate gene expression

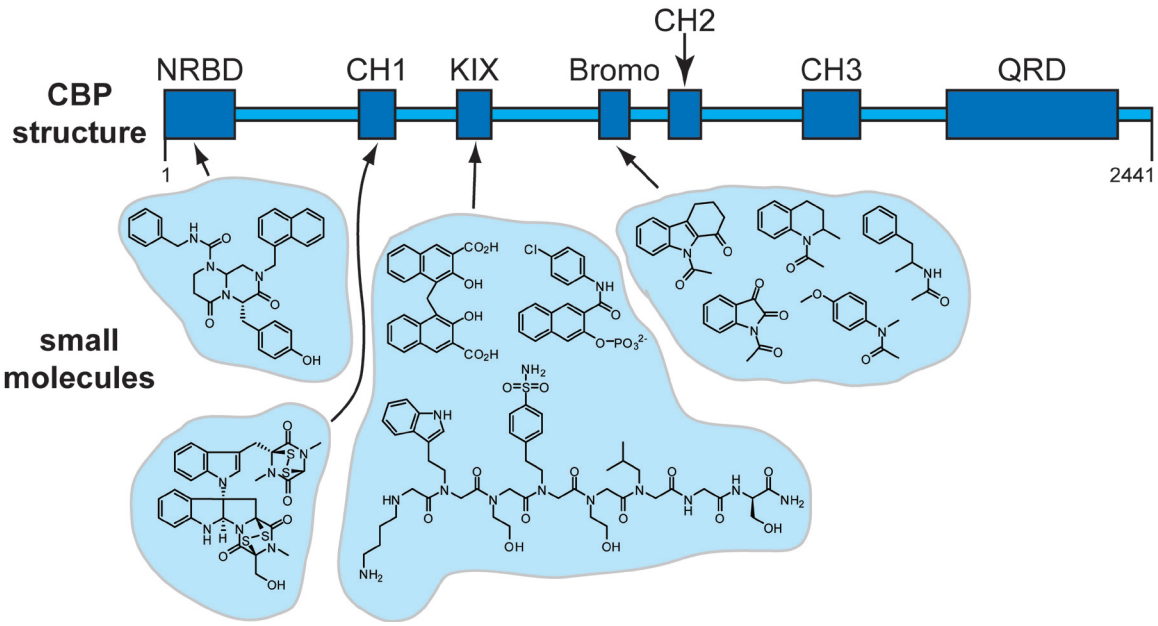

Figure 5.1 The binding sites on the large (~265 kDa) co-activator CREB binding protein (CBP) for several small molecules.

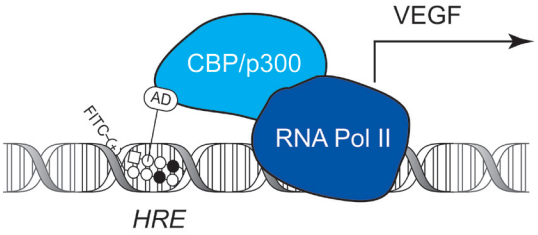

Figure 5.2 Design of a small-molecule mimic of HIF-1α. A DNA-binding polyamide that binds the hypoxia response element (HRE) is conjugated to a CBP-binding small molecule intended to recruit RNA Polymerase II and activate VEGF gene transcription.

(Figure 5.2).

Polyamide conjugates have been shown to act as artificial activators of transcription.[20,23-26] Several peptide activation domains have been shown to be able to activate gene expression *in vitro*.[23-25] Wrenchnolol, a small molecule that binds to the Sur-2 co-activator, has also been shown to serve as an activation domain.[26] Recently, polyamide-peptoid conjugates that were designed to recruit CBP have been reported to modestly activate a transiently transfected luciferase gene in cell culture.[20] This chapter describes conjugates between putative small-molecule activation domains (AD) **1**-**3** which are known to bind to CBP at different sites (Figure 5.3). Each compound has been modified (**4**-**6**) to allow conjugation to the polyamide CtPyPyIm-γ-PyImPyPy. Fluorescent analogues were

found to localize to the nucleus of living cells in the presence of verapamil. A couple of conjugates were also shown to bind the HRE of the VEGF promoter by DNase I footprinting. None of the polyamide conjugates reproducibly activated VEGF expression in cell culture. Surprisingly, the ADs **1** and **3** by themselves were found to activate VEGF gene expression.

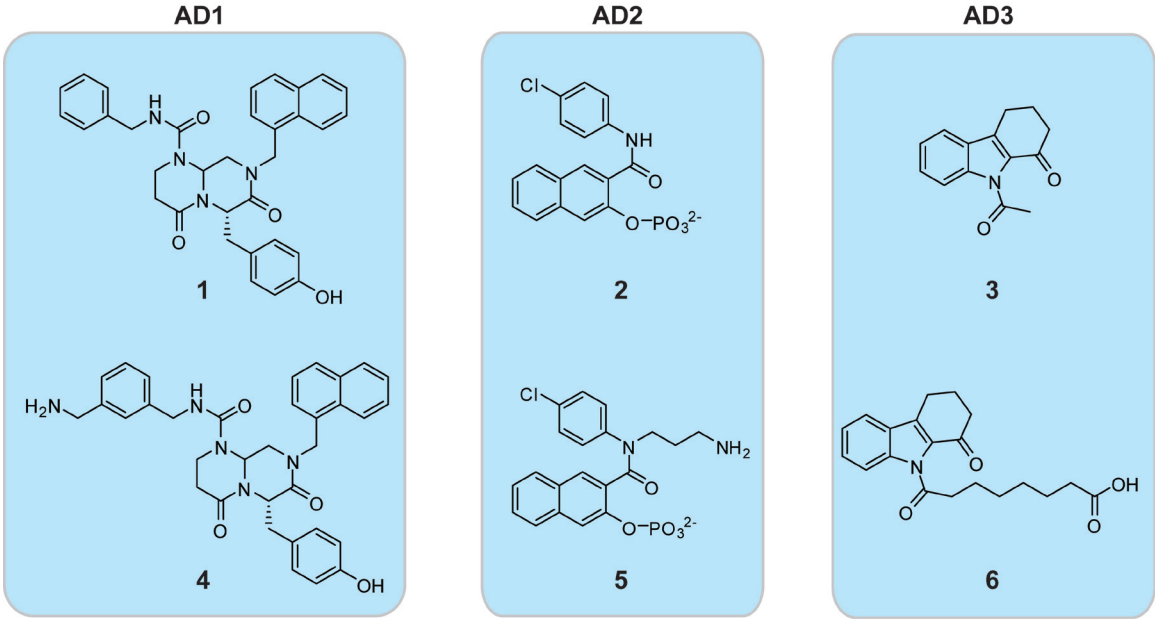

Figure 5.3 Small molecules that bind CBP used as putative activation domains (i.e., AD1 - AD3). The top row shows compounds (**1**-**3**) that were originally reported to bind CBP. The lower row shows compounds (**4**-**6**) that were modified so that they can be attached to a polyamide.

5.2 Synthesis of Small Molecule Activation Domains

Before conjugating to polyamides, the CBP-binding small molecules needed to be synthesized. The compounds **1**-**3** were chosen for their efficacy and synthetic accessibility. ICG-001 (**1**) binds to the nuclear receptor binding domain (NRBD) of CBP and inhibits the CBP/β-catenin interaction with an IC_{50} of 25 μM in cell culture.[17] KG-501 (**2**) binds to the kinase inducible domain (KIX) of CBP and inhibits KID/KIX complex with a K_i of approximately 90 μM.[18] MS7972 (**3**) binds to the bromodomain (BRD) of CBP with a dissociation constant of 19.6 ± 1.9 μM.[19]

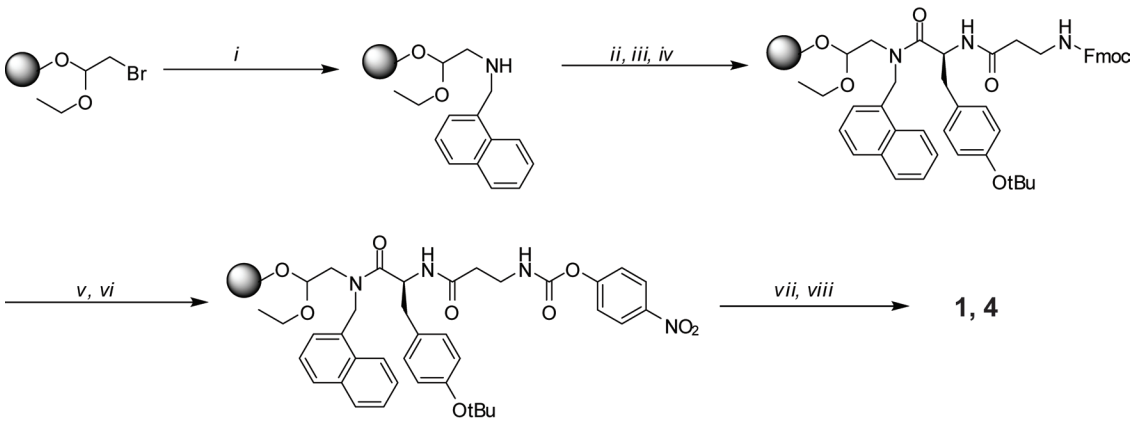

Figure 5.4 Synthesis of AD1 (i.e., **1** and **4**): *i*) 1-naphthalenemethylamine, DMSO, *ii*) Fmoc-Tyr-OH, DIC, HOAt, *iii*) 20% piperidine in DMF, *iv*) Fmoc-βAla-OH, DIC, HOBt, *v*) 20% piperidine in DMF, *vi*) 4-nitrophenyl chloroformate, *vii*) benzylamine or *m*-xylylenediamine, *viii*) formic acid, 20 °C, ~24 hours

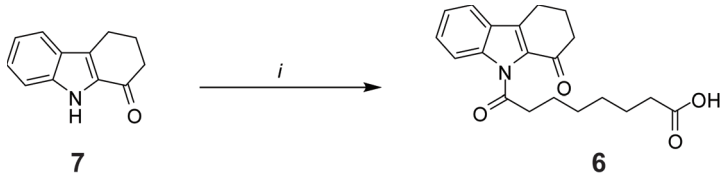

Figure 5.5 Synthesis of AD3 (i.e., **6**): *i*) suberic anhydride, NaH, DMSO

The synthesis of AD1 (**1** and **4**) was performed on solid-phase using bromoacetal resin as described previously (Figure 5.4).[17,27-29] Briefly, the resin-bound bromine was displaced with a naphthylamine and two cycles of standard Fmoc peptide chemistry were performed to add tyrosine and β-alanine amino acids. Following urea formation with either benzylamine or *m*-xylylenediamine, treatment with formic acid led to concomitant resin cleavage and tandem cyclization reactions to yield **1** and **4**, respectively. The synthesis suitably protected analogues of AD2 (**2** and **5**) was performed by Mike Brochu.[30] Also, the synthesis of AD3 (**3**) with a suitable linker (**6**) was performed in one step from a commercially available carbazole **7** by acylating with suberic anhydride (Figure 5.5).

5.3 Rationale for Linker Attachment Position

In modifying compounds **1**-**3** to yield **4**-**6** to enable attachment to a polyamide, it

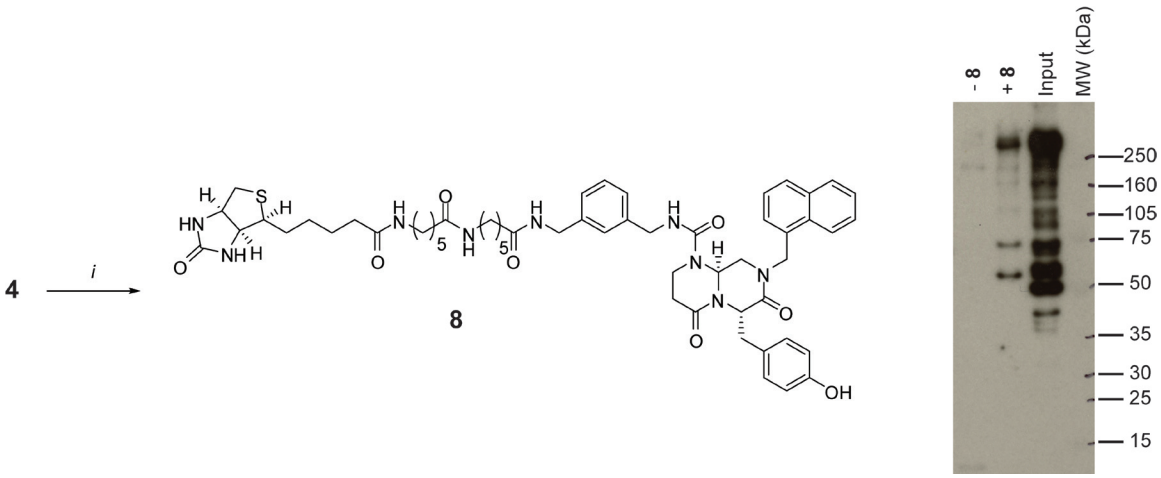

Figure 5.6 Biotin-conjugate pull-down experiments. On the left, synthesis of biotinylated analogoues of AD1 and AD3: *i*) biotinamidohexanoyl-6-aminohexanoic acid *N*-hydroxysuccinimide ester, DIEA in DMF. On the right, a Western blot with a CBP-specific antibody following a pull-down experiment is shown. Biotinylated-AD1 (**8**) is able to bind CBP (~265 kDa) in a mixture of SW480 nuclear extract and maintain this interaction through thorough washing.

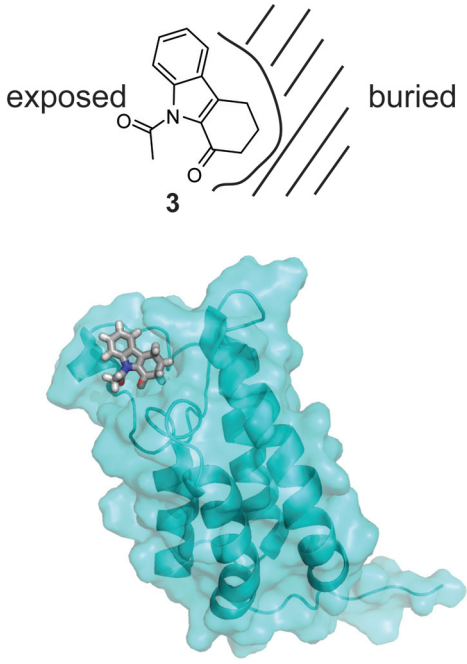

Figure 5.7 NMR structure of the bromodomain of CBP with **3** bound (PDB code 2D82). The acetyl moiety projects away from the binding pocket suggesting this would make a reasonable attachment point.

was unclear whether or not the change would result in elimination of their CBP binding activity. Thus, a pull-down assay was employed to confirm no loss of activity as was previously described for compound **8** (Figure 5.6).[17] First, the biotinylated analogue **8** was synthesized from **4** by treating with a commercially-available biotin succinimidyl ester. A pull-down assay involves treating streptavidin-coated magnetic beads with a biotinylated compound and incubating with input cell lysate which contains a protein of interest. Following thorough washing, the bound protein(s) is(are) eluted by treated with detergent (SDS) and heat (95 °C) and the resulting mixture is separated on an SDS-PAGE gel and Western blotted. Thus, compound **8** was confirmed to pull down CBP from the input SW480 colon cancer cell nuclear extract as evidenced by a band near the expected molecular weight (~265 kDa) that was stained by an anti-CBP antibody (Figure 5.6). Mike Brochu has shown that a biotinylated analogue of **2** is able to pull-down CBP from HeLa cell nuclear extract (data not shown).[30] Although a similar pull-down experiment was not performed for **3** (or **6**), NMR structural data suggests that the acetyl substituent is exposed from the CBP binding pocket[19] so this was chosen as the attachment point (Figure 5.7).

Table 5.1 Frequency of match sites in the VEGF promoter

Sequence	Frequency
WTWCGW	3
WGWWWW	23
WGGWWW	30
WGWGWW	17
WGWWGW	21
WGWWCW	6
WGWCWW	12
WGCWWW	13
WGGGWW	23
WGGWGW	18
WGGWCW	14
WGGCWW	15
WGWGGW	18
WGWGCW	18
WGCGWW	1
WGCWGW	12
WGCWCW	18
WGWCGW	1
WGWCCW	14
WGCCWW	13
WGGGGW	24
WGCGGW	4
WGGCGW	2
WGGGCW	17
WGCCGW	4
WGGCCW	10
WGCGCW	1
WGCCCW	17

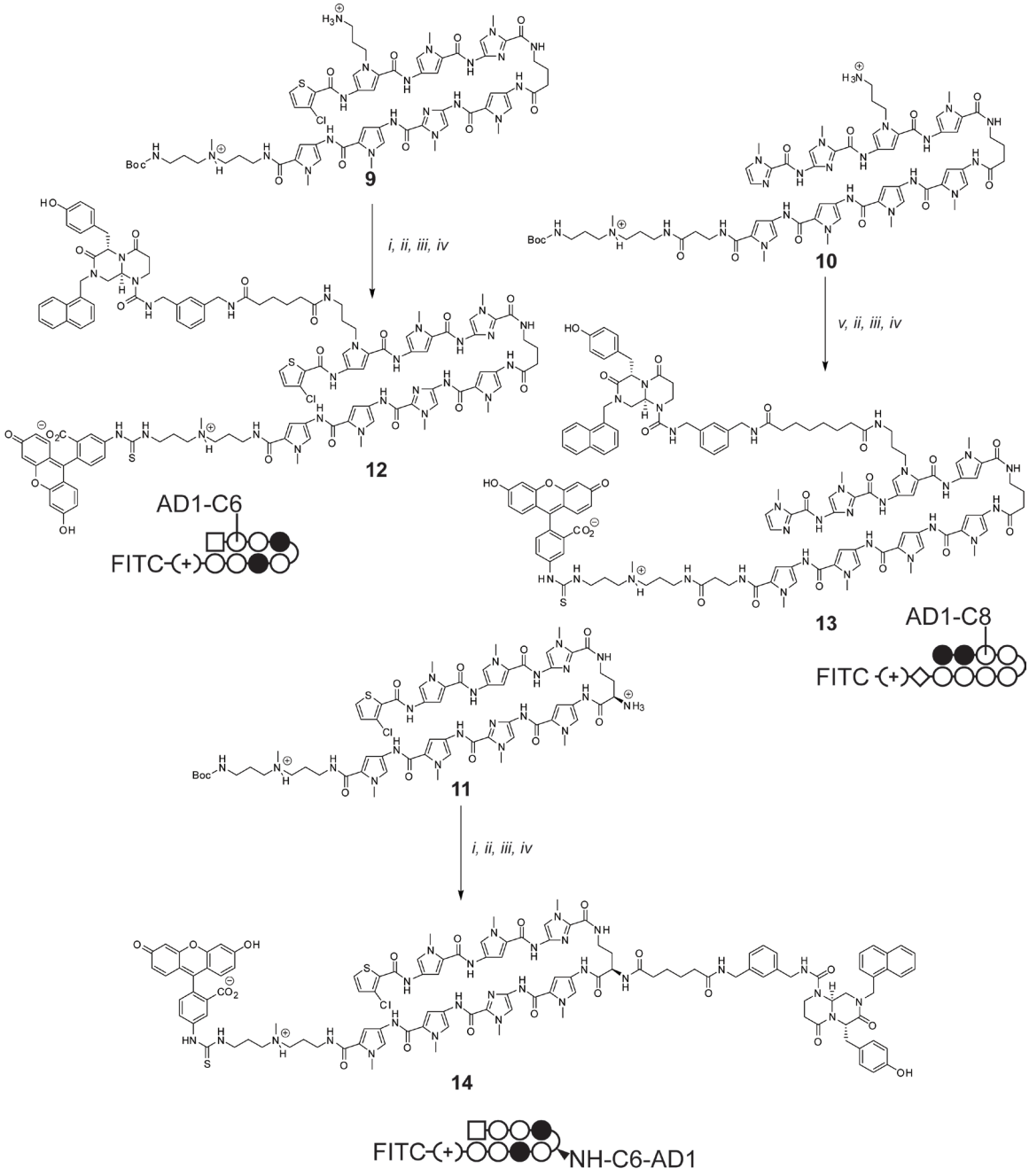

Figure 5.8 Synthesis of polyamide-AD-fluorescein conjugates for cell uptake studies: *i*) excess adipic acid, PyBOP, DIEA, DMF, *ii*) **4**, PyBOP, DIEA, DMF, *iii*) 50% TFA/DCM, *iv*) fluorescein-5-isothiocyante, DIEA, DMF, *v*) suberic anhydride, DIEA, DMF.

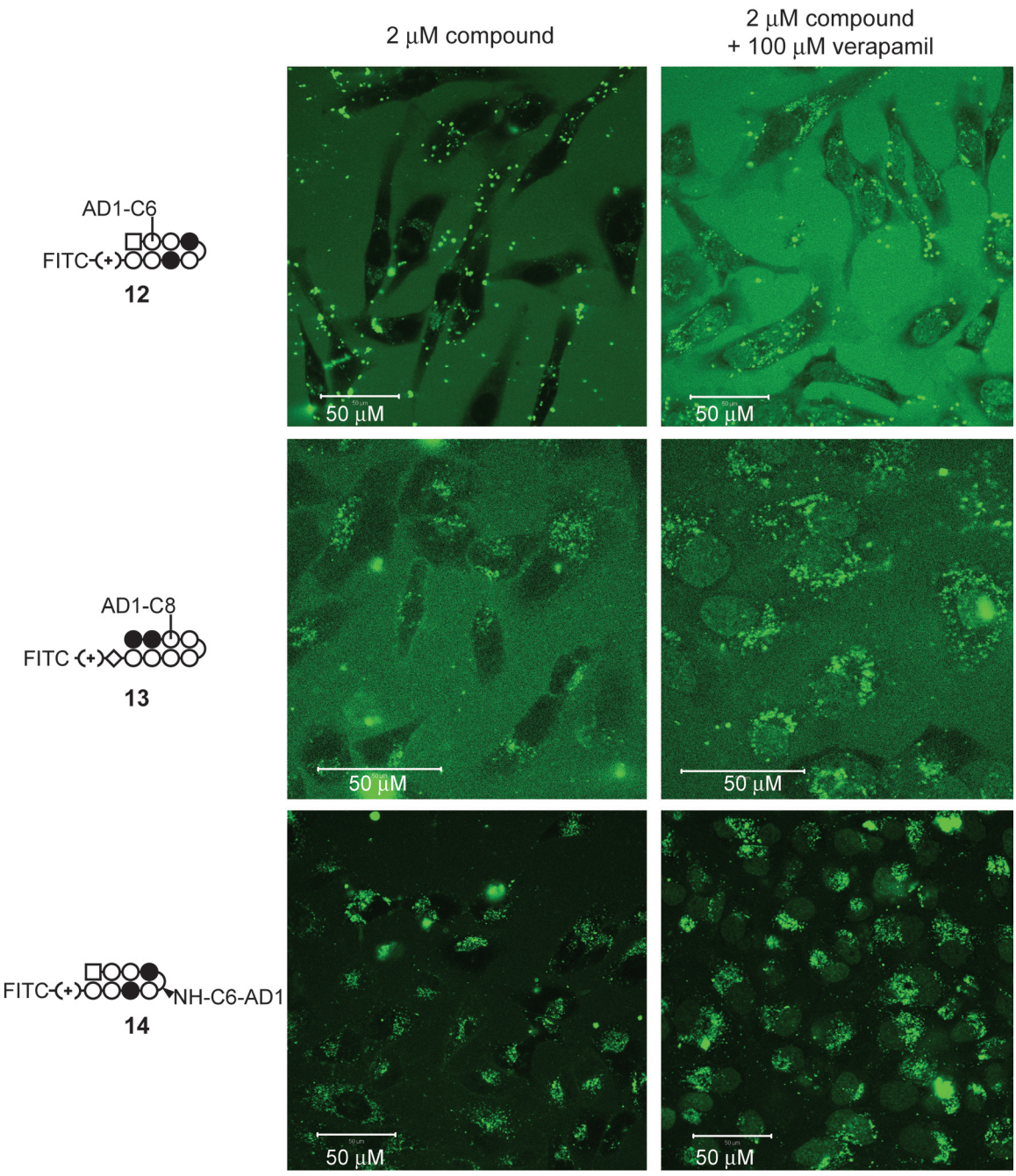

Figure 5.9 Results for uptake experiments in HeLa cells for conjugates **12**-**14**. On the left it is shown that all compounds exhibit extracellular and vesicular staining. On the right it is shown that 100 μM verapamil induces partial nuclear localization. (Scale bar = 50 μm)

5.4 Cell Uptake of Fluorescent Conjugates

To generate compounds suitable for confocal microscopy cell uptake studies, the selectively protected polyamides **9**-**11** were synthesized first by standard methods with Boc-β-Ala-PAM[31] or oxime resin[32] (Figure 5.8). The polyamide of **10** was used because analysis of the VEGF promoter showed a high frequency for its match site 5′-WGGWWW-3′ (Table 5.1). First, a linker was coupled to the polyamide primary amine using either excess adipic acid (6 carbons) or suberic anhydride (8 carbons). Then compound **4** was attached through an amide bond, followed by deprotection, and labeling with fluorescein isothiocyanate to yield **12**-**14**. Confocal microcopy with **12**-**14** in HeLa, MCF-7, and PC-3 cells showed exclusively extracellular and vesicular stains, but the addition of the MDR inhibitor verapamil (100 μM) caused a partial redistribution to the cell nucleus (Figure

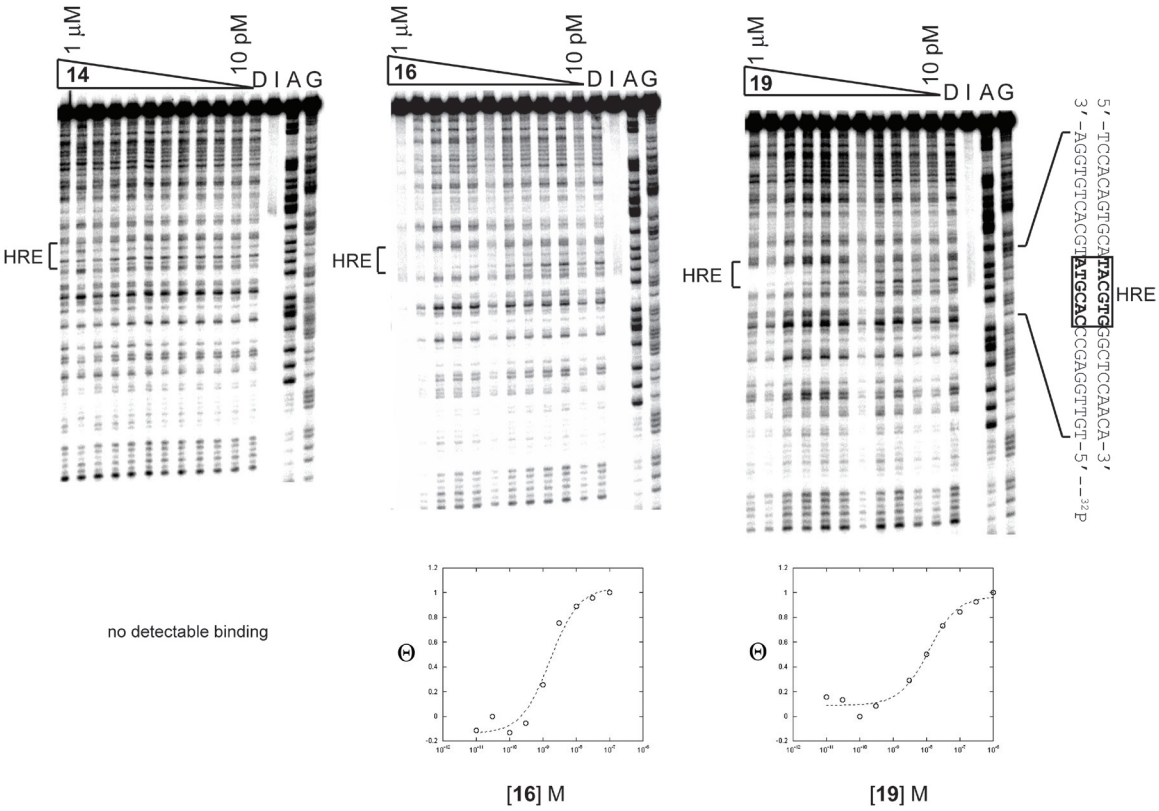

Figure 5.10 Quantitative DNase I footprinting experiments for polyamide-AD conjugates on a stretch of the VEGF promoter which contains the HRE. Compound **14** shows no apparent DNA binding activity (i.e., $K_a \leq 10^6\ M^{-1}$). Compounds **16** and **19** bind to the HRE with K_a's of $6.9 \pm 0.6 \times 10^8\ M^{-1}$ and $8 \pm 5 \times 10^7\ M^{-1}$, respectively.

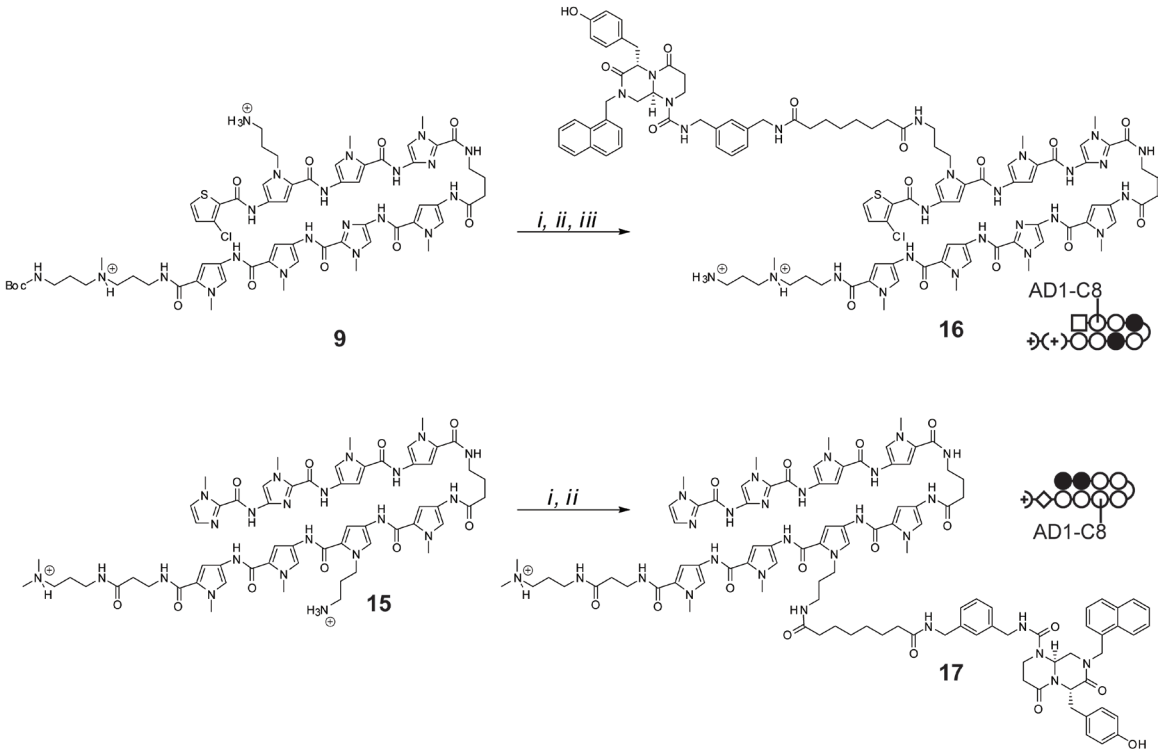

Figure 5.11 Synthesis of *branched* polyamide-AD conjugates: *i*) suberic anhydride, DIEA, DMF, *ii*) **4**, PyBOP, DIEA, DMF, *iii*) 50% TFA/DCM.

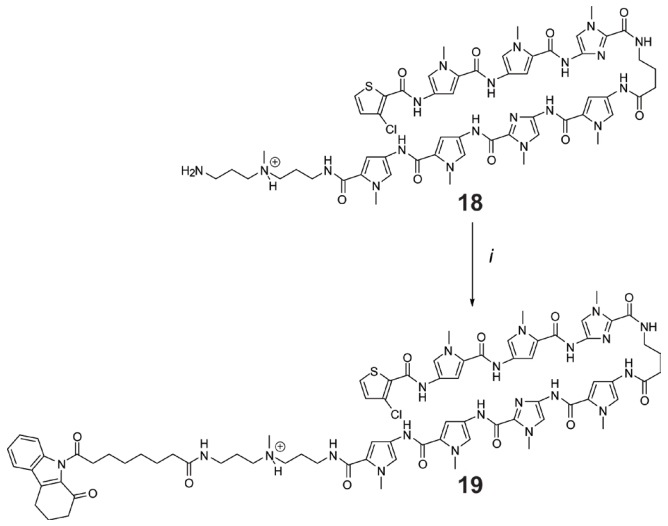

Figure 5.12 Synthesis of *linear* polyamide-AD conjugates: *i*) **6**, PyBOP, DMF, DIEA, 20 °C, 2 hours.

5.9). Titration of verapamil with **13** in HeLa cells suggested that 10 μM verapamil was sufficient to induce nuclear localization so this concentration was used for the RT-PCR described later.

5.5 DNase I Footprinting and Non-Fluorescent Conjugate Synthesis

DNase I footprinting was used to determine the HRE-affinity of polyamide-AD1-FITC conjugate **14** (Figure 5.10). The analogous parent polyamide-FITC conjugate with a free chiral amine at the turn has been reported to have an HRE-affinity of 6.3×10^9 M^{-1} but conjugate **14** shows no apparent DNA binding ($K_a \leq 10^6$ M^{-1}).[21] Attachment of the linker and AD1 appears to be responsible for the reduced DNA affinity. The placement of the linker and AD1 over the three Gs adjacent to the HRE may be particularly detrimental to the binding affinity. Thus, simpler polyamide conjugates with different shapes were designed in hopes they would possess better DNA binding properties (Figures 5.11 and 5.12).

Branched conjugate **16** was synthesized from parent polyamide **10** and small molecule **4** (AD1) after incorporation of an eight-carbon linker (Figure 5.11). The linear conjugate **19** was synthesized from the parent polyamide **18** (Figure 5.12). Compounds **16** and **19** were also footprinted to determine their HRE-binding affinity (Figure 5.10). The presumably dicationic polyamide-AD1 conjugate **16** showed a higher affinity for the HRE ($K_a = 6.9 \pm 0.6 \times 10^8$ M^{-1}) but bound the DNA non-specifically down to a concentration of 300 nM. Linear polyamide-AD3 conjugate **19** revealed a low affinity for the HRE ($K_a = 8 \pm 5 \times 10^7$ M^{-1}). Compound **19**'s affinity would likely be increased by the addition of a chiral amine on the turn.[33]

5.6 Effect of Compounds on VEGF Expression

A total of all six conjugates representing branched and linear configurations were subjected to HeLa cells (i.e., **12**, **13**, **14**, **16**, **17**, and **19**) under non-induced conditions (no

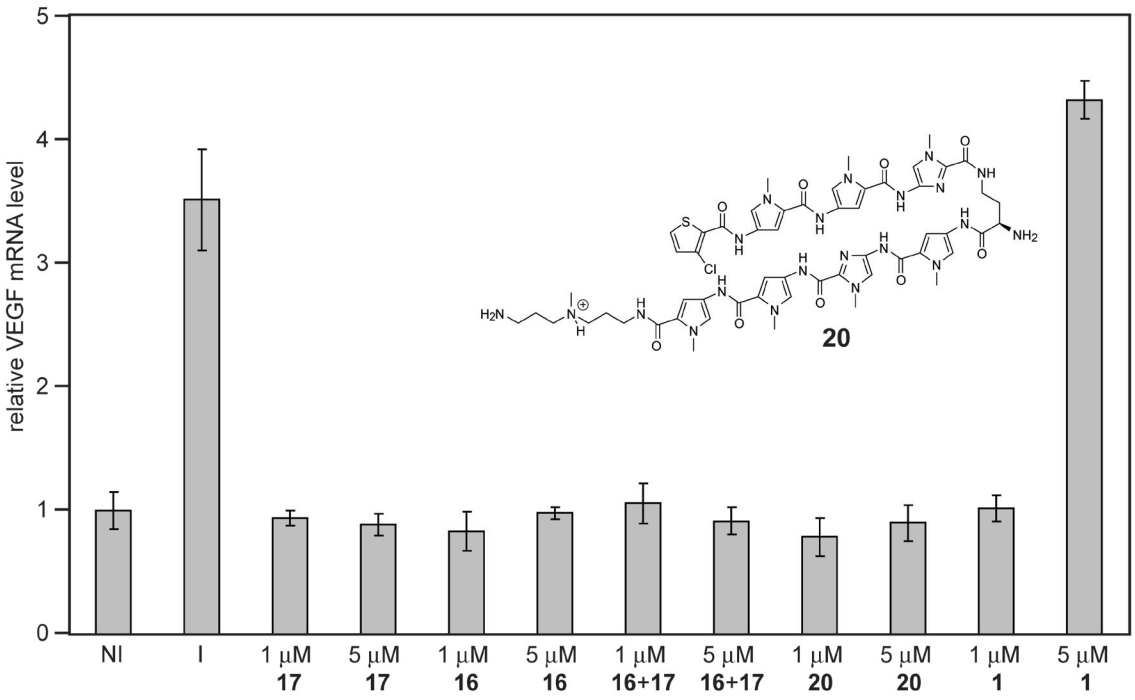

Figure 5.13 RT-PCR experiments measuring the effect of each compound on VEGF expression in the presence of 10 μM verapamil. NI = non-induced baseline control and I = induced (DFO) positive control. Compounds **16** and **17** previously showed weak induction of VEGF gene expression (~1.5-fold increase), but this result was found to be not reproducible as shown here. Combinations of **16** and **17** also do not lead to activation, nor does the polyamide control **20**. Intriguingly, the CBP binding small-molecule **1** by itself activates VEGF gene expression.

DFO) ranging from concentrations of 1 to 15 μM in the presence of 10 μM verapamil. The branched conjugate **17** was also tried since it targets 5′-WGGWWW-3′ which is found with a high frequency in the VEGF promoter (Figure 5.11 and Table 5.1). Quantitative real-time PCR (qRT-PCR) was used to measure the effect of each compound on VEGF gene expression. Of the compounds screened, only **16** and **17** affected VEGF expression (~1.5-fold increase), but this effect was not reproducible (data not shown). Experiments looking for synergistic effects using combinations of conjugates **16** and **17** did not lead to a significant increase in VEGF mRNA levels (Figure 5.13). Remarkably, the small molecules **1** and **3** by themselves, but not **2**, showed a reproducible and significant increase in VEGF mRNA and protein levels (data not shown). This effect by the small molecules themselves was

unanticipated and makes de-convolution of experiments with their conjugates problematic. Investigation into the mechanism of VEGF activation by **1** and **3** is ongoing.

5.7 Experimental Details

Synthesis

ICG-001, AD1 (1). Synthesis was based on described previously described procedures.[17,27-29] Bromoacetal resin resin (333 mg, 0.5 mmol scale, 75-100 mesh, 1% DVB , ~1.5 mmol/g, Aldrich) was loaded with 1-naphthalenemethylamine (1.44 mL) in DMSO (4 mL) for 20 hours at RT. Resin drained and rinsed 2x DMF, 2x DCM, and 2x DMF. Activated Fmoc-Tyr(OtBu)-OH (Novabiochem, 919 mg, 4 eq) with HOAt (AnaSpec, 272.2 mg, 4 eq) and DIC (Aldrich, 310 µL, 4 equivalents) in 5.6 mL of DMF at RT for 20 min before adding to the resin. DIEA (697 µL, 8 equivalents) was added and the reaction was allowed to proceed for 24 hours. Drained and rinsed 2x with DMF. Fmoc-Tyr(OtBu)-OH was coupled again (1.5 eq) for another 24 hours. The partially unreacted resin-bound secondary amine was capped with 10 mL of 4:1 DMF:Ac_2O for 2.5 hours. The resin was rinsed as above. The Fmoc protecting group was removed with 20% piperidine in DMF for 25 min and rinsed as usual. Fmoc-β-Ala-OH (622.6 mg, 4 equivalents) was pre-activated with HBTU (739.5 mg, 3.9 equivalents) and DIEA (522 µL) and coupled to the resin overnight. The resin was rinsed and deprotected as before. 4-nitrophenylchloroformate (503.9 mg, 5 eq) was coupled in DCM with DIEA (522 µL, 6 eq) for 1 hour at RT. Rinsed 2x with DCM and added DMF (~3 mL) and benzylamine (218 µL, 4 equivalents) letting the reaction proceed for 5 hours. Rinsed 2x DMF, 2x DMF, 2x DCM and dried *in vacuo* for 5 min. Cyclization and cleavage was performed with formic acid (88%, 8.7 mL) for 10.5 hours at RT. The resin was filtered with a small plastic filter. The filtrate concentrated under reduced pressure and dissolved in 0.1% TFA in water and acetonitrile, purified by preparative HPLC, and lyophilized to yield 25.3 mg of a white powder (46.1 µmol, 9.2% yield measured by UV). ^{1}H NMR (d_6-DMSO, 300 MHz) δ 2.04-2.09 (m, 2H), δ 3.00-3.15 (m, 3H), δ 3.22-3.28 (m, 1H), δ 3.55 (t, J = 11.4

Hz, 1H), δ 3.84 (m, 1H), δ 4.13-4.20 (dd, J_1 = 6.0 Hz, J_2 = 15.6 Hz, 1H), δ 4.28-4.35 (dd, J_1 = 6.0 Hz, J2 = 15.3 Hz, 1H), δ 4.89 (d, J = 15.3 Hz, 1H), δ 5.12 (d, J = 15.6 Hz, 1H), δ 5.10-5.13 (m, 1H), δ 5.74-5.78 (d of d, J_1 = 3.6 Hz, J_2 = 10.5 Hz, 1H), δ 6.54 (d, J = 8.7 Hz, 2H), δ 6.90 (d, J = 8.7 Hz, 2H), δ 7.21-7.38 (m, 6H), δ 7.44-7.60 (m, 4H), δ 7.87-7.97 (m, 2H), δ 8.12-8.14 (m, 1H), δ 9.18 (bs, 1H); A_{max} at 281 nm (ε = 7,860 $M^{-1}\cdot cm^{-1}$ in 20% acetonitrile in 0.1% TFA/water). (ESI-MS) $[M+H]^+$ calc'd for $C_{33}H_{33}N_4O_4$ 549.2, observed 549.1.

KG-501, AD2 (2). This compound is a common histological stain commercially available from several sources (e.g., Fluka).

MS7972, AD3 (3). This compound is commercially available in small quantities (ChemBridge Corporation, San Diego, CA).

ICG-001-aminomethyl, AD1-NH_2 (4). The synthesis of this compound was performed as described for compound **1** replacing benzylamine with *m*-xylylenediamine (518 μL). After cleavage from resin and HPLC purification 12.6 mg of a white powder was obtained (~6% yield). ^{1}H NMR (d_6-DMSO, 300 MHz) δ 2.06-2.13 (m, 2H), δ 3.01-3.12 (m, 4H), δ 3.57 (t, 1H, J = 10.8 Hz), δ 3.82-3.86 (m, 1H), δ 3.98 (d, 2H, J = 6 Hz), δ 4.16-4.23 (dd, 1H, J_1 = 5.7 Hz, J_2 = 15.15 Hz), δ 4.25-4.32 (dd, 1H, J_1 = 5.7 Hz, J_2 = 15.15 Hz), δ 4.87 (d, 1H, J = 15.3 Hz), δ 5.14 (d, 1H, J = 14.7 Hz), δ 5.12-5.17 (m, 1H), δ 5.72 (dd, 1H, J_1 = 3.9 Hz, J_2 = 10.8 Hz), δ 6.54 (d, 2H, J = 8.7 Hz), δ 6.90 (d, 2H, J = 8.7 Hz), δ 7.21 (d, 1H, J = 7.5 Hz), δ 7.28-7.40 (m, 4H), δ 7.44-7.62 (m, 4H), δ 7.89 (d, 1H, J = 8.1 Hz), δ 7.94-7.97 (m, 1H), δ 8.11 (bs, 2H), δ 8.11-8.14 (m, 1H), δ 9.18 (s, 1H); HRMS (FAB) exact mass calcd for $C_{34}H_{36}N_5O_4$ requires 578.2767 *m/z*, found 578.2754 *m/z*.

MS7972-suberic acid, AD3-CO_2H (6). First, 2,3,4,9-tetrahydro-1H-carbazol-1-one **7** (150 mg, 0.81 mmol, 1 eq, Bionet Research Intermediates) was dissolved in DMSO (1 mL, anhydrous) and sodium hydride (64.8 mg, 1.62 mmol, 2 eq, 60% oil dispersion) was added as a solution in DMSO (1 mL). The solution was sonicated to aid dissolution. After 5 min, suberic anhydride (151.7 mg, 0.97 mmol, 1.2 eq) was added as a solution in DMSO (1.5 mL). Immediately a precipitate formed and the solution was poured into 10% citric acid

buffer and extracted 3x with DCM (50 mL). The organic layers were combined, dried with anhydrous sodium sulfate, filtered, and dried under reduced pressure to give a thin orange film. The desired product was purified by silica gel chromatography (1:1 EtOAc:Hexanes + 0.1% acetic acid, $R_f \approx 0.3$) and dried under reduced pressure. The resulting film was dissolved in MeOH (500 μL) and added to water to form an off-white precipitate which was flash frozen and lyophilized (19.11 mg, 7% yield). ^{1}H NMR (d_6-DMSO, 300 MHz) δ 1.20 (pent, J = 3.6 Hz, 4H), δ 1.40 (m, 2H), δ 1.60 (m, 2H), δ 2.10-2.20 (m, 4H), δ 2.64 (t, J = 6.9 Hz, 2H), δ 2.86 (t, J = 7.2 Hz, 2H), δ 3.00 (t, J = 6.0 Hz, 2H), δ 7.33 (t, J = 7.8 Hz, 1H), δ 7.53 (t of d, J_1 = 1.2 Hz, J_2 = 8.4 Hz, 1H), δ 7.79 (d, J = 8.1 Hz, 1H), δ 7.97 (d, J = 8.7 Hz, 1H), δ ~12 (bs, 1H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 189.78, 178.89, 175.82, 139.60, 139.33, 132.38, 130.32, 126.71, 123.82, 121.45, 115.93, 39.74, 39.40, 34.02, 29.07, 28.96, 25.72, 24.79, 23.69, 22.38. HRMS (FAB) exact mass calcd for $C_{20}H_{24}NO_4$ requires 342.1705 *m/z*, found 342.1717 *m/z*. Absorbance maximum at 310 nm in water with 2% DMSO (ε at 308-309 nm = 2.2 ± 0.8 x 10^4 $M^{-1}cm^{-1}$).

ICG-002, AD1-biotin (8). An aliquot of **4** was dissolved in 200 μL of 0.1 M DIEA in DMF and 2.3 mg of biotinamidohexanoyl-6-aminohexanoic acid *N*-hydroxysuccinimide ester (Sigma) was added. The product formed immediately and was purified by preparative HPLC and lyophilized to yield a fine white powder (1.5 μmol). (MALDI-TOF MS) Calcd for $C_{56}H_{72}N_9O_8S^+$ 1030.5, observed 1031.0.

CtPy$^{(propylamine)}$PyIm-γ-PyImPyPy-(+)-NHBoc (9). This polyamide was synthesized on Marshall-Liener resin and purified by preparative HPLC (Appendix D). (MALDI-TOF MS) Calcd for $C_{63}H_{82}ClN_{21}O_{11}S^+$ 1375.6, observed 1375.7.

ImImPy$^{(propylamine)}$Py-γ-PyPyPyPy-β-(+)-NHBoc (10). This polyamide was synthesized on PAM resin (1.94 μmol, 3% overall yield). (MALDI-TOF MS) Calcd 1408.7, observed 1408.7.

CtPyPyIm-γ$^{NH2(R)}$-PyImPyPy-(+)-NHBoc (11). This polyamide was synthesized by Nick Nickols on oxime resin and purified by preparative HPLC. (MALDI-TOF MS) Calcd for

$C_{61}H_{77}ClN_{21}O_{11}S^+$ 1347.6, observed 1346.5.

CtPy$^{(AD1\text{-}C6\text{-}propylamine)}$PyIm-γ-PyImPyPy-(+)-FITC (12). This conjugate was synthesized essentially as described below for **14**. (ESI MS) Calc'd $[M+2H]^{2+}$ $C_{119}H_{125}ClN_{27}O_{20}S_2^{2+}$ 1175.9, observed 1176.2.

ImImPy$^{(AD1\text{-}C8\text{-}propylamine)}$Py-γ-PyPyPyPy-β-(+)-FITC (13). Polyamide **10** was coupled to suberic anhydride as described for compound **17** and the resulting intermediate was purified by preparative HPLC and lyophilized (609 nmol, 31% yield over 1 step). (MALDI-TOF MS) Calcd 1564.8, observed 1564.7. The Boc group was removed and the FITC coupled as described below for **14**. The final product was purified by preparative HPLC and lyophilized. (ESI MS) Calcd $[M+2H]^{2+}$ 1207.5, observed 1207.3.

CtPyPyIm-γ$^{(AD1\text{-}C6\text{-}NH2(R))}$-PyImPyPy-(+)-FITC (14). Polyamide **11** (1.55 μmol) was treated with excess adipic acid (10 eq) and PyBOP (20 eq) in ~300 μL of 0.1 M DIEA in DMF. The resulting intermediate was purified by preparative HPLC and lyophilized (560 nmol, 36% yield for 1 step). (MALDI-TOF MS) Calcd for $C_{67}H_{84}ClN_{21}O_{14}SNa^+$ 1496.6, observed 1496.6. The previous intermediate was activated with PyBOP (6.7 mg) in 600 μL 0.1 M DIEA in DMF and added to amine **4**. The compound was precipitated from diethyl ether (10 mL) cooled over dry ice, centrifuged, and dried briefly with $N_{2(g)}$. The Boc protecting group was removed with 50% TFA/DCM (1 mL) for 30 min at RT and the compound was precipitated from ether and dried as above. The pellet was redissolved in 1 M DIEA in DMF (600 μL) and fluorescein-5-isothiocyanate (2 μmol) was added. The final product was purified by preparative HPLC and lyophilized to give a yellow-orange solid (179 nmol, 32% final 2 steps). (ESI MS) Calc'd $[M+2H]^{2+}$ $C_{117}H_{122}ClN_{27}O_{20}S_2^{2+}$ 1162.42, observed 1162.1.

ImImPyPy-γ-PyPy$^{(propylamine)}$PyPy-β-Dp (15). This polyamide was synthesized on Boc-β-Ala-PAM resin. (MALDI-TOF MS) Calcd for $C_{60}H_{77}N_{22}O_{10}S^+$ 1265.6, observed 1265.6.

CtPy$^{(AD1\text{-}C8\text{-}propylamine)}$PyIm-γ-PyImPyPy-(+)-$NH_3^+$ (16). Suberic anhydride (3 eq) was reacted with polyamide **9** as described for compound **17** below. The intermediate was

purified by preparative HPLC and lyophilized (624 nmol, 40% yield over 1 step.) (MALDI-TOF MS) Calcd 1530.7, observed 1530.5. Amine **4** was coupled to this intermediate as described for **14** and the final product was purified by preparative HPLC (193 nmol, 31% yield over 1 step). (MALDI-TOF MS) Calcd 1990.9, observed 1990.9.

ImImPyPy-γ-PyPy$^{(AD1\text{-}C8\text{-}propylamine)}$PyPy-β-Dp (17). Polyamide **15** (1.4 μmol) was treated with suberic anhydride (1.5 eq) in 200 μL 0.1 M DIEA in DMF and heated to 35 °C for 1 hour. The intermediate was purified by preparative HPLC and lyophilized to give a white powder (421 nmol, 30% yield). (MALDI-TOF MS) Calcd for $C_{68}H_{89}N_{22}O_{13}^+$ 1421.7, observed 1422.1. The previous intermediate was activated with PyBOP (10 equivalents) in 600 μL 0.1 M DIEA in DMF and added to amine **4** (1.5 equivalents). The final product was purified by preparative HPLC and lyophilized to give a white powder (145 nmol, 36% yield over 1 step). (MALDI-TOF MS) Calcd for $C_{102}H_{122}N_{27}O_{16}^+$ 1981.0, observed 1980.7.

CtPyPyIm-γ-PyImPyPy-(+)-NH$_3^+$ (18). This polyamide was synthesized on oxime resin. (MALDI-TOF MS) Calcd 1231.5, observed 1231.4.

CtPyPyIm-γ-PyImPyPy-(+)-C$_8$-AD3 (19). Polyamide **18** (1.19 μmol) was coupled to **6** (0.85 mg, 2.5 μmol) using PyBOP (6 μmol) in a solution of 0.1 M DIEA in DMF (200 μL) and purified by preparative HPLC and lyophilized (561 nmol, 47 % yield). (MALDI-TOF MS) Calcd 1554.6, observed 1554.7. The extinction co-efficient of the conjugate was assumed to be the sum of the individual components (i.e., ε at 310 nm of 91,500 $M^{-1}cm^{-1}$= 69,500 $M^{-1}cm^{-1}$ + 22,000 $M^{-1}cm^{-1}$).

CtPyPyIm-γ$^{NH2(R)}$-PyImPyPy-(+)-NH$_3^+$ (20). This compound was synthesized and characterized by Nick Nickols.

Pull-down assay and Western blot. The pull-down was based on a previously published experiment.[17] 30 μL of 10 mg/mL Dynabeads M-280 Streptavidin coated (Invitrogen) were aliquoted into an eppendorf tube to which 4 nmol of biotin conjugate **8** was added as a 1:1 solution of DMSO:PBB for a final volume of 30 μL. (PBB = 20 mM HEPES,

100 mM NaCl, 0.5 mM EDTA, 6 mM $MgCl_2$, 0.5% Nonidet P-40 (Tergitol, Type NP-40, Sigma), 1 tablet complete protease mixture (Roche), 5 mM β-mercaptoethanol added immediately before use, pH 7.9, total volume 50 mL) Beads incubated overnight and then washed 3x with PBB. Added 28.5 µL of PBB and 1.5 µL of SW480 nuclear extract (1.88 mg/mL Bradford concentration) obtained previously from Pierce Nuclear Extract Kit (Cat # 78833) and allowed to incubate for ~2 hours at RT. The tubes were put on ice for 10 min and washed 3x with cold PBB in the cold room. Added PBS:Reducing Agent: LDS 9.1:1.4:3.5 and heated to 90 °C for 5 min and ran on an SDS-PAGE gel (Invitrogen, NuPAGE 10% bis-TRIS gel). The resulting gel was transferred to a membrane and blotted with a primary anti-CBP antibody (1:200, A-22, sc-369, Santa Cruz Biotechnology) and a secondary goat anti-rabbit HRP (1:5000, Santa Cruz Biotech). The resulting gel was visualized with SuperSignal West Pico (Pierce) and developed with a 90-second exposure to light-sensitive film in the dark room.

Cell culture and confocal microscopy experiments. The cell culture and confocal microscopy experiments were performed as described in chapter 4. Racemic verapamil (Aldrich, 98%) was added at the same time as the conjugates.

DNase I footprinting. Footprinting was performed as described in Chapter 2 using a previously published plasmid of a region of the VEGF promoter containing the HRE.[21]

Quantitative real-time PCR (qRT-PCR). These experiments were performed by Nick Nickols essentially as described previously.[21,34-35]

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