Chapter 5

Inhibition of Transcription on the Androgen Response Element with Polyamides and Polyamide Conjugates

Abstract

Upon binding an androgen molecule, androgen receptor (AR) acts as a transcriptional activator on genes bearing one of the several androgen-response elements (ARE) in the 5' upstream promoter region. The prostate specific antigen (PSA) gene is among several genes regulated through an ARE. The AR-ARE interaction is inhibited upon treatment with minor groove binding polyamides targeted to the ARE site. Inhibition of AR binding to the ARE is expected to decrease the transcription of PSA, which is shown in a transient transfection assay utilizing a luciferase gene under control of an ARE. Attempts to extend these results to native gene transcription in live cells were unsuccessful. Polyamide-fluorescein conjugates of the active polyamides were found to spontaneously enter the nuclei of LNCaP cells. These conjugates were also found to bind the ARE sequence-specifically and with high affinity. Preliminary inhibition results show only modest inhibition with the polyamide-fluorescein compounds. However, higher concentrations and/or treatment with multiple compounds may show increased PSA inhibition activity. The synthesis of higher-affinity compounds that target the same DNA sequence in the ARE would also likely effect higher transcription inhibition results.

Introduction

The androgen receptor (AR) is a ligand-activated transcription factor that is part of the steroid hormone receptor superfamily of proteins.[1-3] By binding their specific receptor proteins, steroid hormones act to coordinate many complex series of events involved in the development, differentiation, and physiological response of cells to a variety of stimuli. Upon binding a steroid hormone molecule, due to allosteric reorganization receptor proteins become able to bind short (~20 bp) *cis*-acting hormone receptor elements (HRE).[4] AR action is initiated upon binding of testosterone (T) and, especially, 5α-dihydrotestosterone (DHT). The AR regulates gene expression upon binding to androgen-response elements (ARE) located in the 5' flanking region of androgen target genes.

Many genes are regulated through ARE domains, including some found in kidney, liver, and especially prostate tissue. Chief among the latter is the prostate specific antigen (PSA), which is frequently used as a marker for the diagnosis of prostate cancer. The androgen receptor, like the similar glucocorticoid and progesterone receptors, binds its response element as homodimers.[5] The study of AR-regulated genes has produced a consensus ARE sequence: 5'-GGWACAnnnTGTTCT-3', along with other related nonconsensus sequences. The PSA ARE domain is a nonconsensus 5'-AGAACAgcaAGTGCT-3' sequence.[6] From crystal structure studies, it was shown that the capitalized hexamer sequences are key to the interaction of the AR with its DNA binding site (Figure 5.1).[7,8] Though the protein interacts mainly with the major groove, each unit of the homodimer injects a lysine residue into the minor groove. Based on the

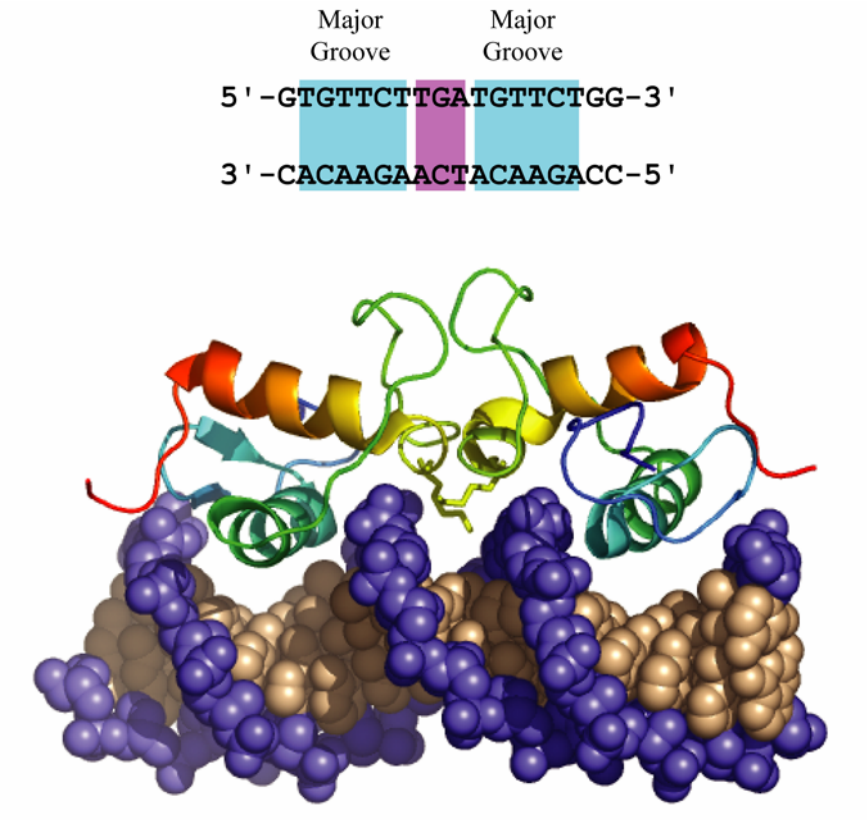


Figure 5.1 Androgen receptor binding DNA. The two homodimeric AR chains bind the consecutive hexameric half-site direct repeat 5'-ACAAGA-3' units utilizing mainly major groove contacts (blue-green helices lying in the major grooves). Each AR molecule projects a lysine (yellow) into the minor groove.[8]

structural and biochemical data, polyamides were synthesized and tested for their ability to inhibit the binding of AR to ARE.

Results

Polyamides **1-3** were synthesized based on their projected ability to bind the PSA ARE domains (Figure 5.2). These compounds were sent to Dr. Zhengxin Wang at the M.D. Anderson Cancer Center at the University of Texas Southwestern for testing as inhibitors of AR-ARE interaction. Gel mobility shift assays[9] showed promising results, with compounds **1** and **2** showing complete inhibition of mobility at concentrations ≤ 1

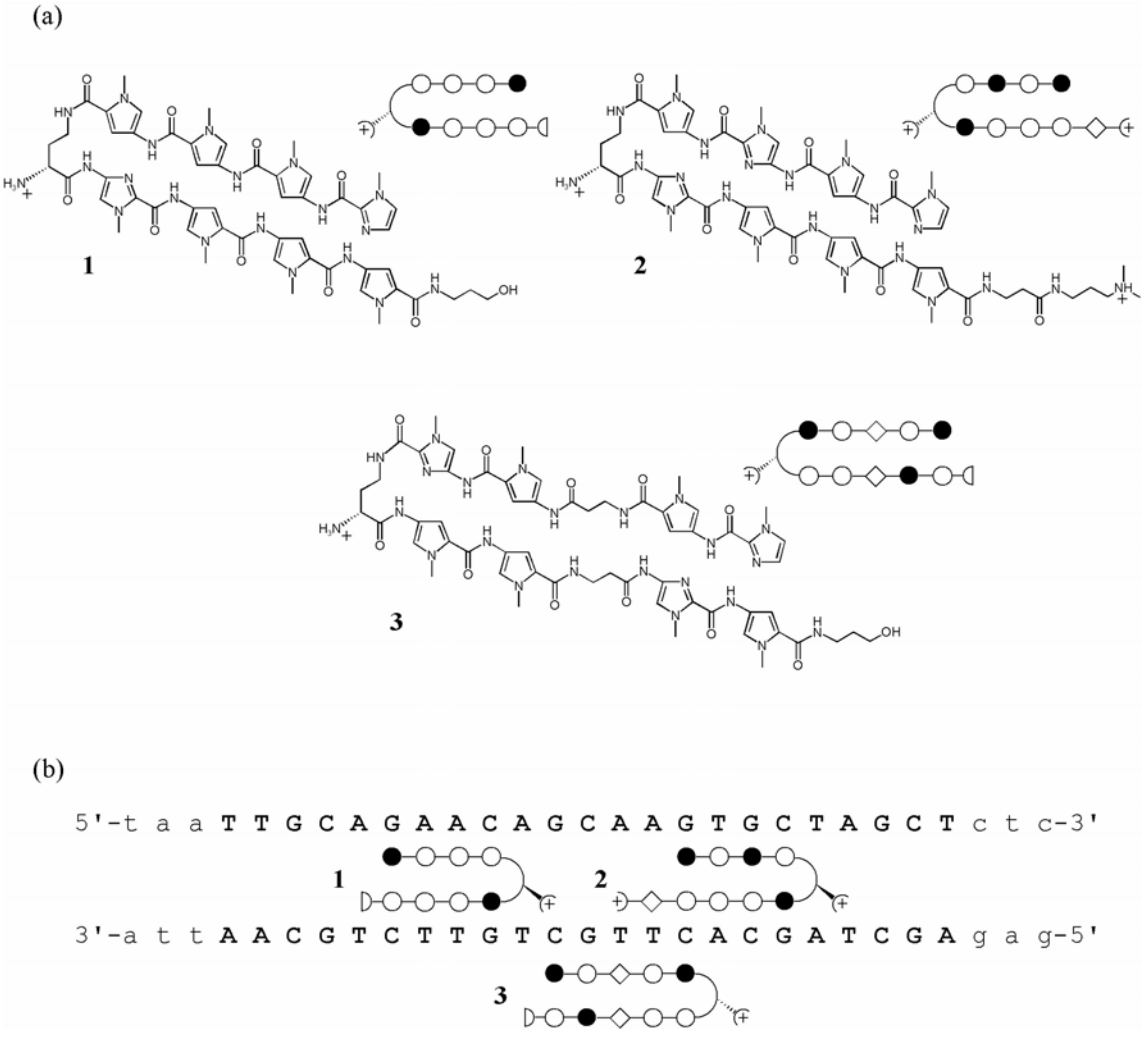


Figure 5.2 Compounds for AR inhibition. (a) Chemical structures and ball-and-stick models of compounds **1**-**3**. (b) Putative binding sites for **1**-**3** on the PSA ARE (in boldface).

nM. Compound **3** also inhibits AR-ARE interaction, but at higher concentrations (≥ 4 nM). Based on these positive results, the compounds were tested in DNase I footprinting titration assays using a plasmid containing the PSA promoter (Figure 5.3). These compounds were then tested in an *in vitro* transcription assay using a DNA construct containing a luciferase gene under transcriptional control of a PSA ARE. The polyamides **1**-**3** also showed strong inhibition in this system (Figure 5.4).

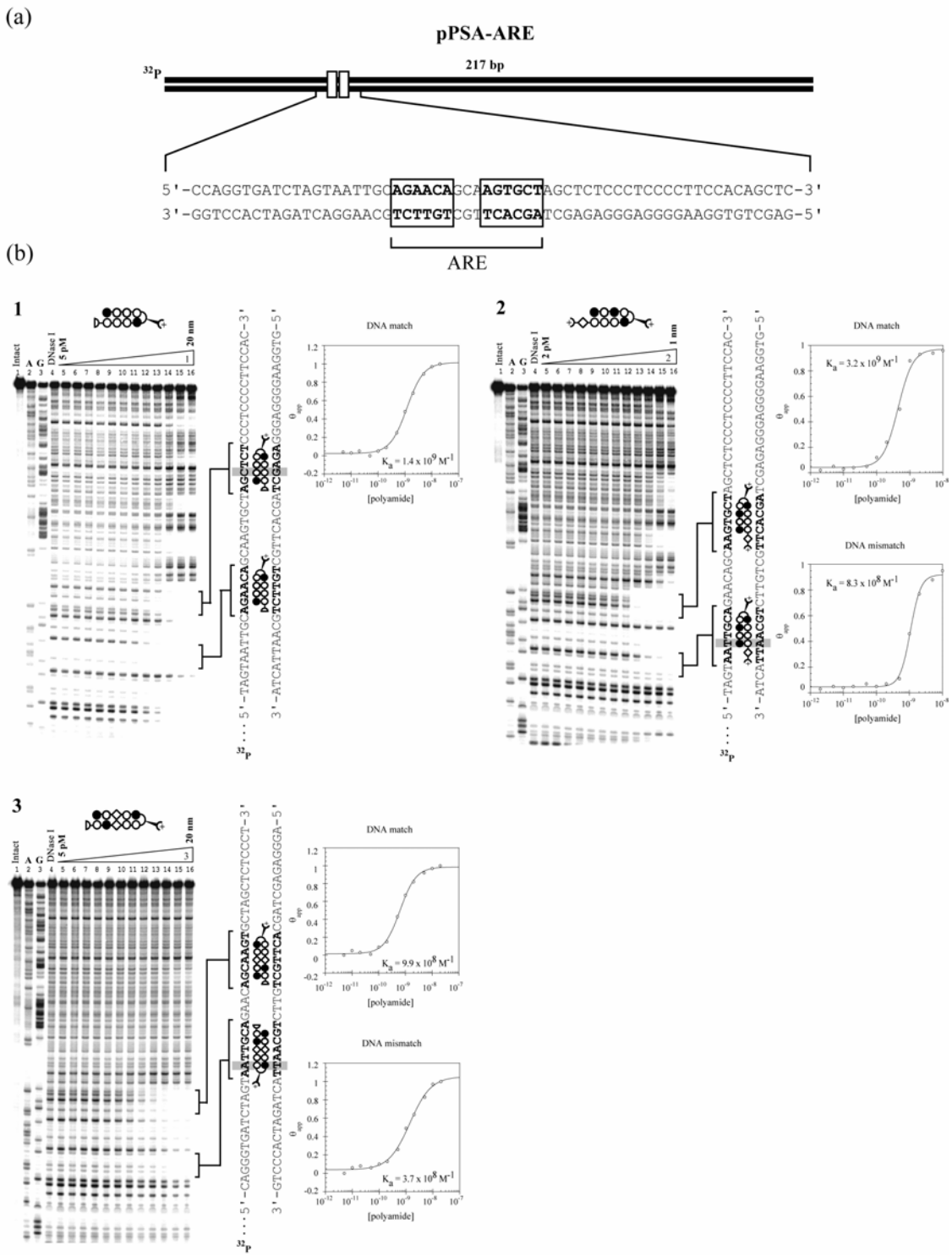
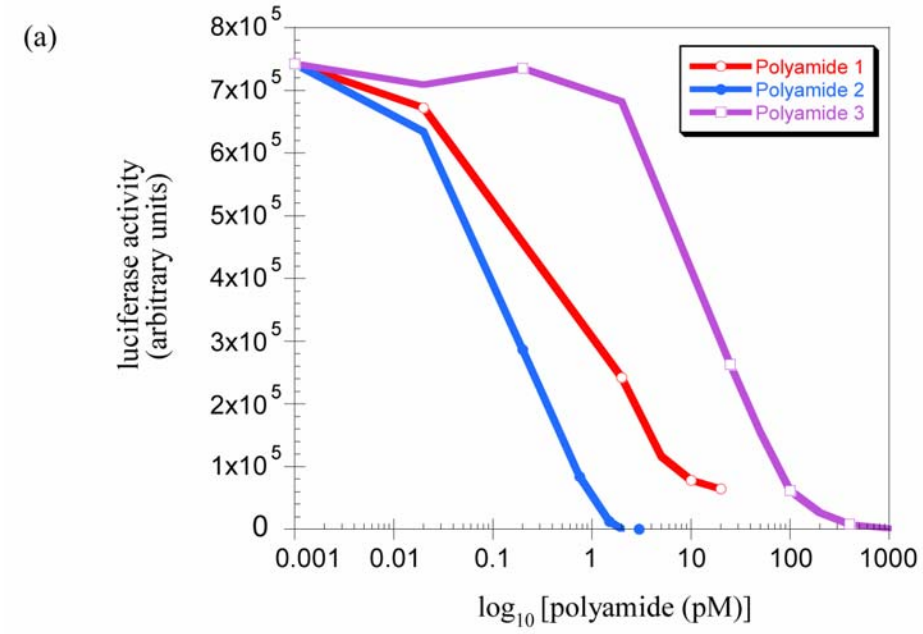


Figure 5.4 DNase I Footprinting of **1-3**. (a) PSA promoter PCR fragment used for footprinting, bearing the ARE. (b) Footprinting gels and isotherms for compounds **1-3**.



(b)

	K_a (M^{-1})	IC_{50} (pM)
1	1.4 x 10^9	2
2	3.2 x 10^9	0.2
3	9.9 x 10^8	25

5′-A A T T G C A G A A C A G C A A G T G C T A G C T C T-3′
1
3′-T T A A C G T C T T G T C G T T C A C G A T C G A G A-5′

5′-A A T T G C A G A A C A G C A A G T G C T A G C T C T-3′
2
3′-T T A A C G T C T T G T C G T T C A C G A T C G A G A-5′

5′-A A T T G C A G A A C A G C A A G T G C T A G C T C T-3′
3
3′-T T A A C G T C T T G T C G T T C A C G A T C G A G A-5′

ARE

Figure 5.4 *In vitro* transcription assay for the inhibition of AR action by polyamides **1-3**. (a) The dose response curves for polyamides **1-3** are depicted showing luciferase activity as a function of polyamide concentration. Polyamide **2** shows a markedly lower IC_{50} than the other polyamides (0.2 pM, blue line.) Polyamide **1** reaches IC_{50} at 2 pM (red line). Polyamide **3** is much less potent, reaching IC_{50} at 25 pM (violet line). (b) Summary of DNA-binding and AR-inhibition characteristics of polyamides **1-3**. Though there is < 5-fold difference in binding affinity between **2** and **3**, there is a > 100-fold increase in AR inhibition, showing that polyamide position is a critical variable in successful inhibition of transcription.

In attempts to inhibit the AR-ARE interaction *in vivo*, transient transfection assays were performed in LNCaP and PC3 prostate cancer cell lines using an ARE-containing luciferase reporter. PC3 cells do not contain endogenous AR, so one may probe the activity of AR with precision by adding controlled amounts of AR. However, this also means that one cannot study the inhibition of an endogenous PSA gene via the polyamide binding of ARE. LNCaP cells contain AR and the PSA gene is actively transcribed in the cell. The advantage LNCaP cells provide is the ability to study the effect of polyamides inhibiting an endogenous gene through ARE binding by performing Western blots or RT-PCR experiments to analyze gene products (either protein or mRNA, respectively.) Unfortunately, all attempts to use polyamides **1**-**3** to inhibit gene expression *in vivo* failed to produce any positive results.

Speculating that the negative results were due to the inability of polyamides to localize to genomic DNA, either by exclusion from the cell or sequestration in cytoplasmic vesicles, polyamide conjugates were synthesized to promote uptake into live LNCaP and PC3 nuclei. The first such compounds synthesized were conjugates of polyamides with DHT (**4**-**9**, Figure 5.5). The synthesis of a representative polyamide-DHT conjugate is shown in Figure 5.6. These were designed to take advantage both of the recognition of steroid hormones by cell membrane transport proteins, to bring the polyamides in to the cell, and the DHT-recognition and nuclear transport properties of AR, to transmit the polyamides into the cell nucleus (Figure 5.7).[10-12] Compounds **4**-**7** were based on polyamides **1** and **2**, which showed the strongest inhibition *in vitro*. They incorporate the DHT moiety, linked by a short polyethylene glycol (PEG) domain, in two different positions on the polyamide, the tail and the *N*-methyl position of an internal

pyrrole residue. Compounds **8** and **9** are based on a double pairing-rule mismatch polyamide of both **1** and **2**, incorporating the DHT moiety as **4-7**.

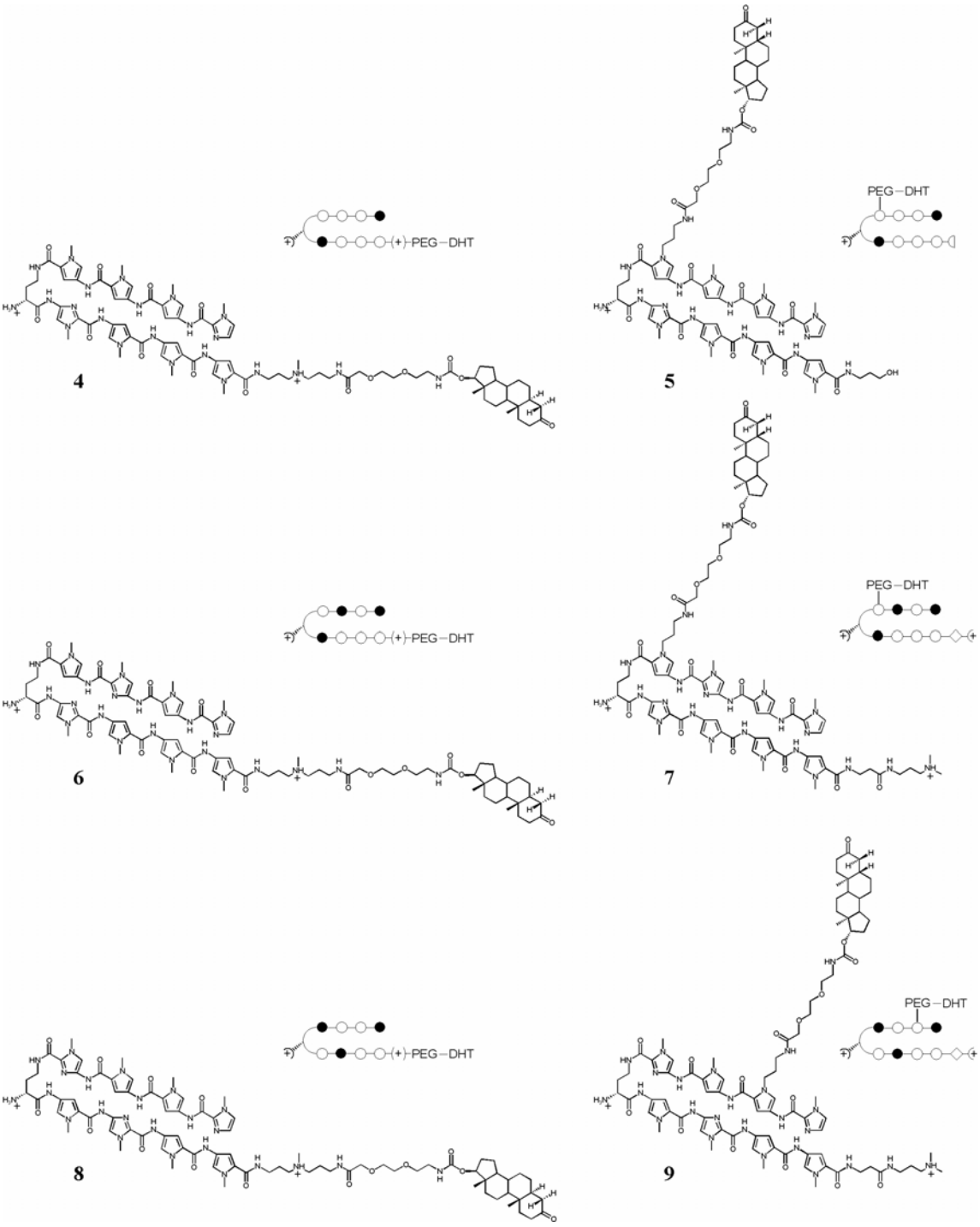


Figure 5.5 Polyamide-DHT conjugates for AR inhibition. Chemical structures and ball-and-stick models of compounds **4-9**.

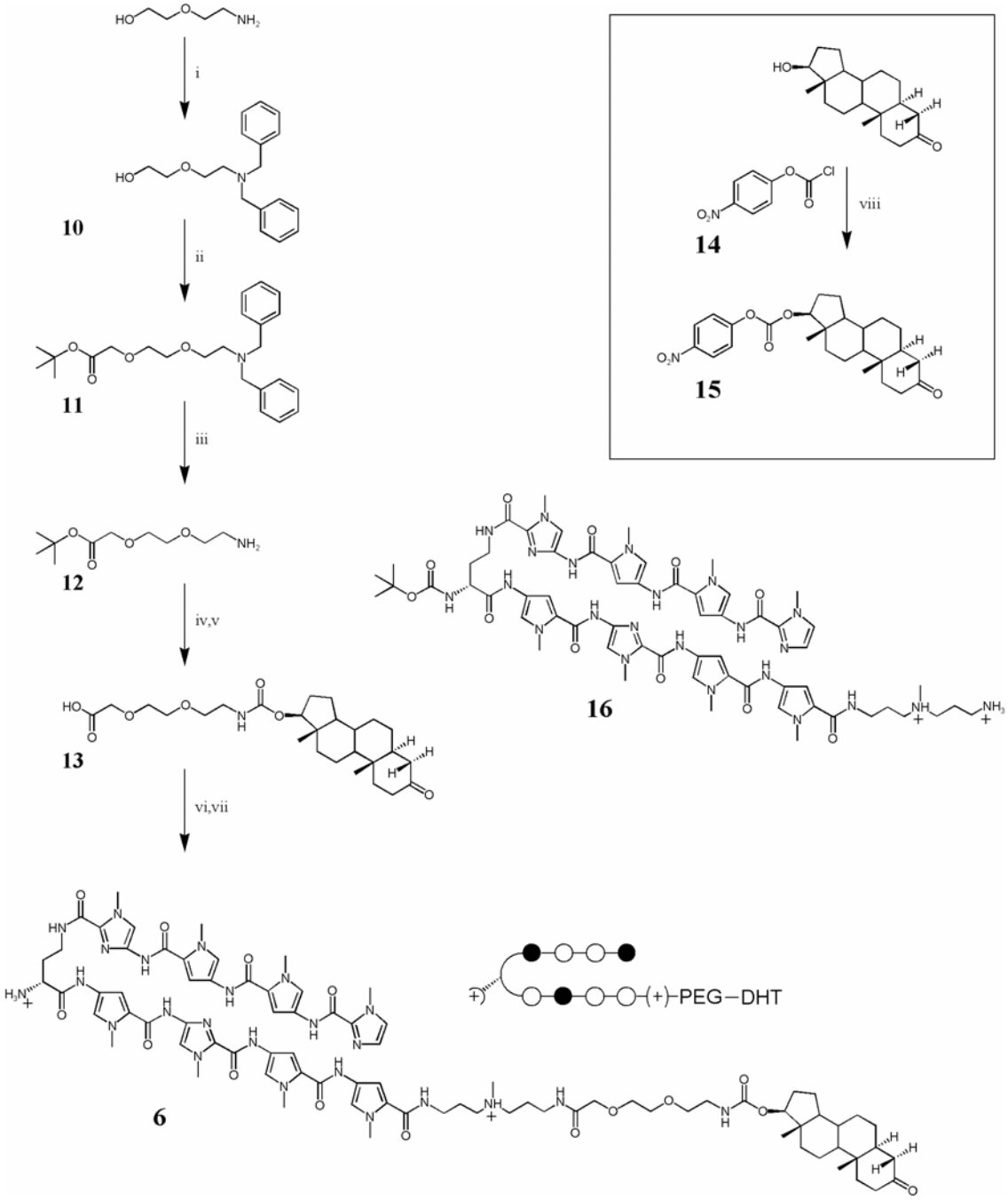


Figure 5.6 Synthesis of polyamide-DHT conjugates. (i) benzyl bromide, K_2CO_3, H_2O. (ii) *t*-butylbromoacetate, NaH, DMF, 0ºC. (iii) Pd/C (10% wt/wt), H_2, MeOH, AcOH. (iv) **15**, NEt_3, CH_2Cl_2, 60ºC. (v) 50% (v/v) TFA/CH_2Cl_2, 1hr at room temperature. (vi) **16**, DCC, HOBt, DIEA, DMF. (vii) 20% TFA/CH_2Cl_2, 1hr at room temperature. (viii) *p*-nitrophenylchloroformate (**14**), NEt_3, CH_2Cl_2.

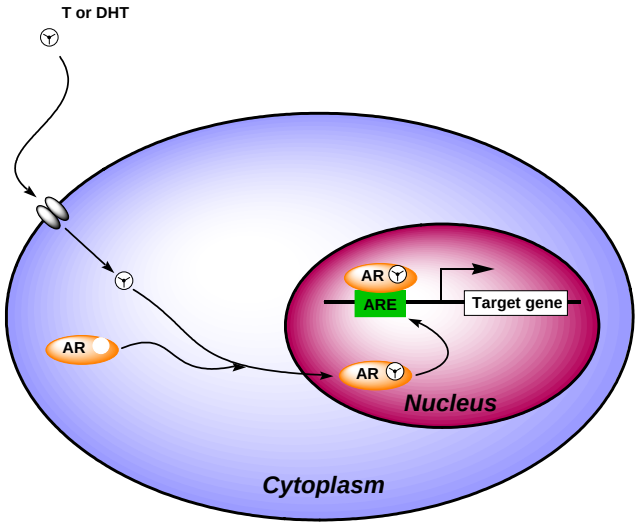


Figure 5.7 Mechanism of androgen-initiated AR-mediated transcription activation. Androgen, here T or DHT, passes through the cytoplasmic membrane either through diffusion or active transport. In the cytoplasm it binds AR, which translocates to the nucleus. Upon recognition of the ARE site, androgen-AR complex (along with other chaperone proteins) binds ARE stimulating transcription of the target gene.

In order to test the effect on DNA-binding of conjugating the DHT-linker moiety to a polyamide, conjugates **4** and **5** were footprinted on the same PSA-ARE PCR fragment as **1**-**3** (Figure 5.8a). Overall the addition of the DHT-linker moiety reduces the affinity of the polyamide for its match site ~10-fold with reference to the parent polyamide **1**. Attachment off of the tail also erodes the ability of the compound to differentiate between DNA sites, lowering its specificity. Attachment off of the *N*-methyl position of an internal pyrrole does not have the same negative effect on DNA specificity.

Compounds **4**-**9** were used in a transient transfection assay in PC3 cells, which lack endogenous AR. The cells were treated with AR and 1μM DHT-polyamides. The effect on transcription of a luciferase gene under control of ARE sites was measured by luciferase activity (Figure 5.8b). Conjugate **4** was the most effective, showing ~45% reduction in luciferase activity. Compound **5**, also based on polyamide **1**, was less

effective, showing 25% reduction. The only other conjugate to show a significant effect was **6**, based on polyamide **2**, which showed ~20% reduction in luciferase. The higher level of inhibition of **4** and **5** over **6** and **7** is somewhat surprising, since the *in vitro* results suggested compounds based on polyamide **2** should be the most efficient inhibitors. This may be a result of higher nuclear transport of **4** and **5** over **6** and **7**.

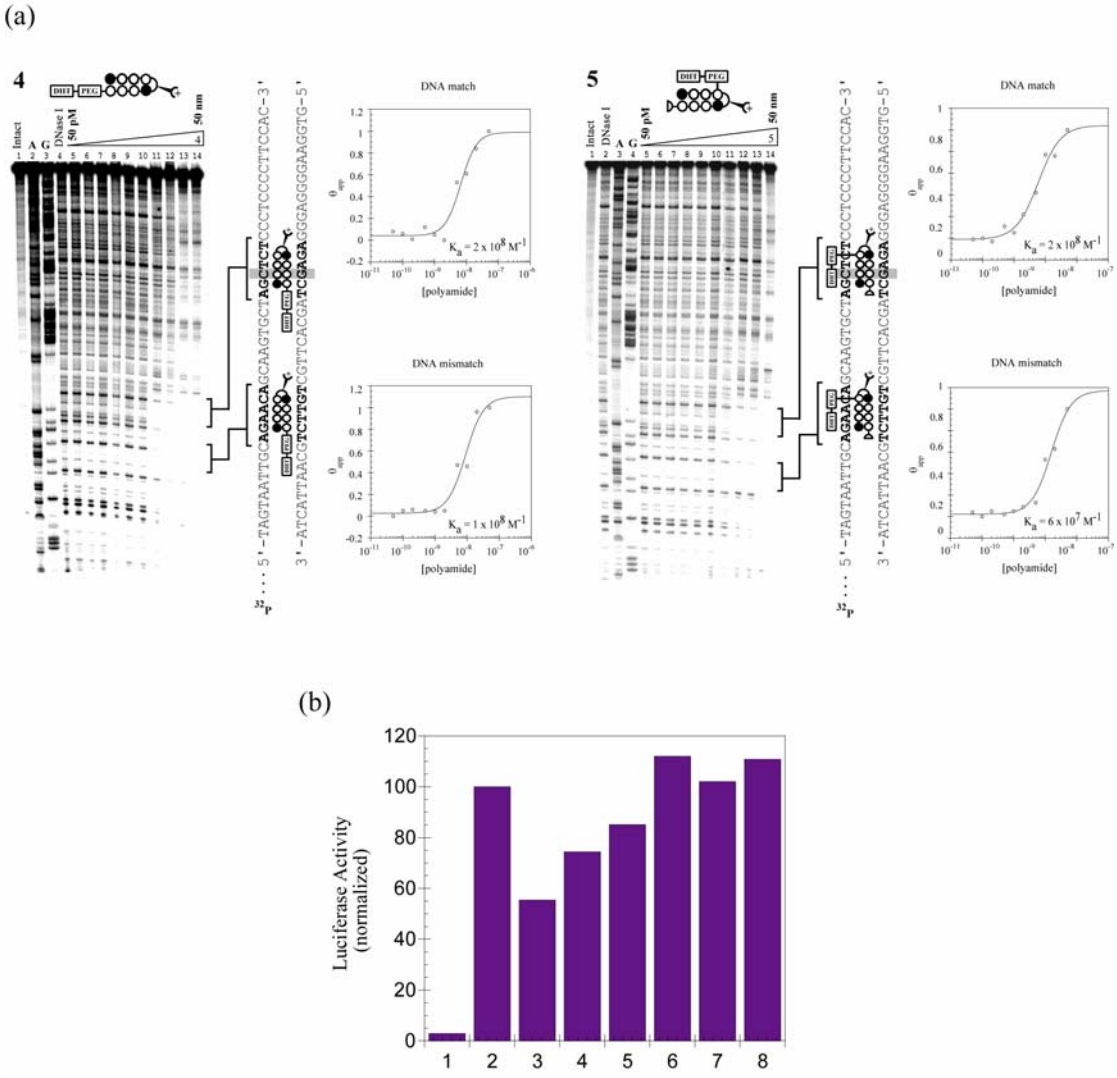


Figure 5.8 DNA-binding and transcription inhibition characteristics of DHT conjugates. (a) DNase I footprinting gels and isotherms for **4** and **5**. (b) Inhibition of luciferase transcription was greatest for compound **4**, at ~45% reduction (activity normalized to +AR –PA sample. Key: column 1: –AR, -PA; column 2: +AR, -PA; column 3: +AR, 1 μM **4**; column 4: +AR, 1 μM **5**; column 5: +AR, 1 μM **6**; column 6: +AR, 1 μM **7**; column 7: +AR, 1 μM **8**; column 8: +AR, 1 μM **9**.

After it was determined that polyamide-fluorescein conjugates tended to possess favorable uptake qualities (see Chapter 4), fluorescein derivatives **17**-**19** were prepared (Figure 5.9a). These compounds were designed based on polyamides **1** and **2**, as well as a double pairing-rule mismatch with reference to **1** and **2**. After verification that they stained the nuclei of PC3 and LNCaP cells, the conjugates were used in the same transient transfection assay that was used on compounds **4**-**9** (Figure 5.9b). Compound **17** seemed to show some inhibition at 0.1 μM. However, compound **18** did not show any significant inhibition. As expected, mismatch compound **19** also produced no inhibitory effect. Since it was shown that these three compounds all enter cell nuclei, it is surprising that the compounds were less efficient than even the DHT conjugates at inhibiting transcription. It may be that the DNA-binding affinities of **17** and **18** are low enough that higher concentrations (> 1μM) are needed. Also, a combination of treatment with both **17** and **18** might provide greater inhibition than either one alone could, in a synergistic fashion.

Outlook and Future Directions

The success in creating DNA-binding polyamides with the ability to cross the cytoplasmic membrane and localize to genomic DNA provides the basis for undertaking many *in vivo* transcription regulation projects. In the case of AR-ARE inhibition, the *in vivo* experiments performed to date have all been on model systems transiently transfected into cells. However, the ultimate goal of transfection experiments with DNA-binding polyamides is the inhibition of endogenous genes upon polyamide treatment. To this end it would be interesting to treat cells that actively transcribe the PSA gene under

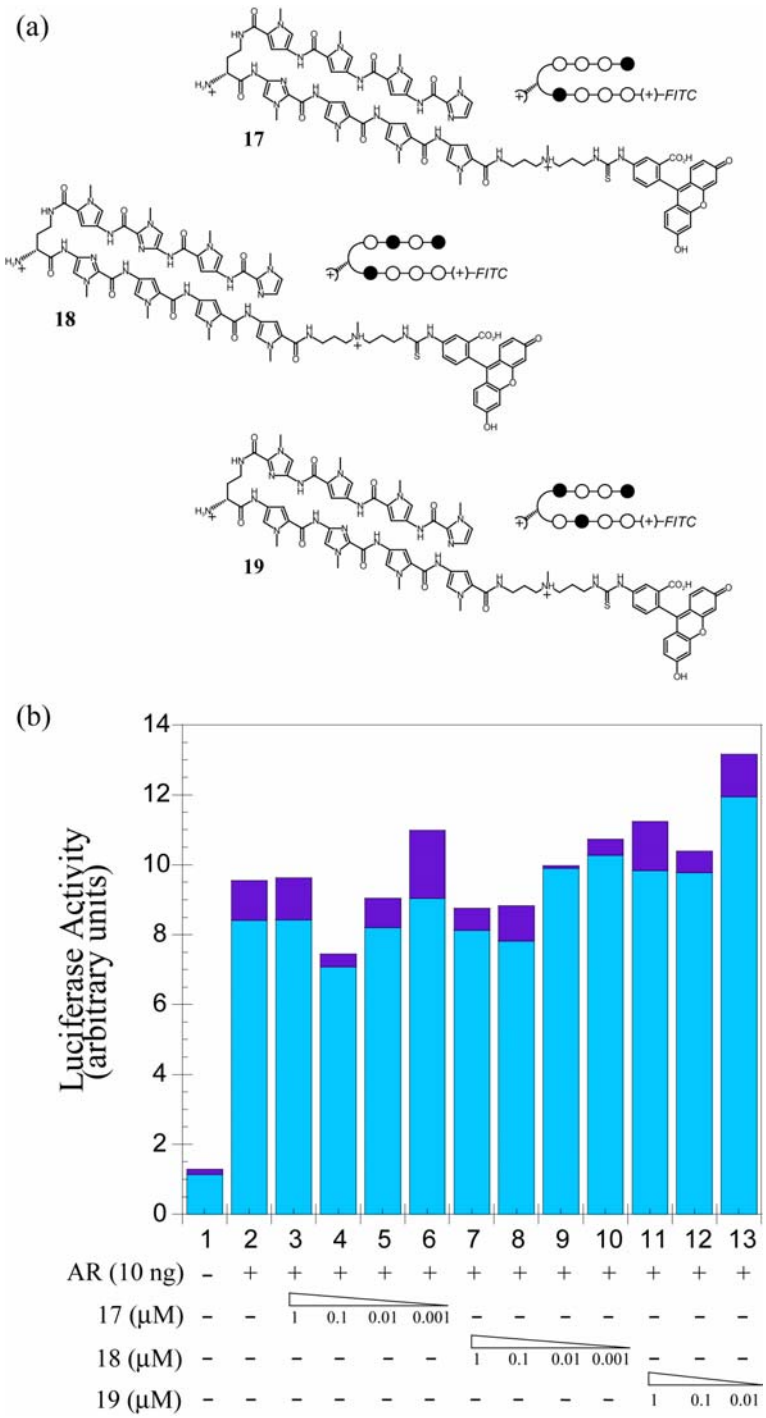


Figure 5.6 Polyamide-FITC conjugates for AR-ARE inhibition. (a) Chemical structures and ball-and-stick models for **17**-**19**. (b) Transient transfection luciferase inhibition assay. Compound **17** seems to have some small effect on luciferase transcription, though strangely not at the highest concentration (1 μM, column 3, compare to column 2). Compounds **18** and **19** show no significant effect. The light blue portion of the bars is luciferase activity, in arbitrary units. The dark blue portion is ½ of the standard deviation after three replicate experiments.

control of AR, such as LNCaP cells, and assay for changes in the level of gene products, either protein (Western blot) or mRNA (RT-PCR). The development of new aromatic heterocycles that can take part in DNA recognition will permit the targeting of DNA sequences in a more general manner. By combining new polyamides based on the lead compounds **1** and **2**, with uptake vectors such as FITC, and by assaying their effects on endogenous PSA production by RT-PCR, it seems likely that a path to successful gene inhibition in living cells by polyamides will be illuminated.

Experimental

Chemicals

Polyamides were synthesized by solid phase methods on Boc-β-ala-PAM resin (Peptides International, Louisville, KY)[13] or on Kaiser oxime resin (Nova Biochem, Laufelfingen, Switzerland).[14] All fluorescent dye reagents were from Molecular Probes. Chemicals not otherwise specified were from Sigma-Aldrich.

Synthesis of Polyamide-DHT Conjugates

2-(*N*,*N*-Dibenzyl-2-aminoethoxy)ethanol (10)[15]

2-(2-aminoethoxy)ethanol (15 mL, 150 mmol), potassium carbonate (41.5 g, 300 mmol), and 45 mL water were added to a 250 mL round bottom flask equipped with a magnetic stirrer and a dropping funnel. Benzyl bromide (35.7 mL, 300 mmol) was added dropwise over 1 hr. The solution was then stirred overnight at room temperature. Et_2O (250 mL) was added to the mixture, separated, and the aqueous layer was washed with a further 50 mL Et_2O. The combined organic portions were washed with 300 mL brine and

150 mL water, then dried with $MgSO_4$ and concentrated to yield a clear oil. This was purified on a short column of silica with 5:1 hexanes:EtOAc→100% EtOAc to yield a clear oil (22 g, 52% yield). ^{1}H-NMR ($CDCl_3$) δ 7.20-7.38 (m, 10 H), 3.65 (s, 4 H), 3.58 (t, 2 H), 3.47 (t, 2 H), 2.69 (t, 2 H), 2.39 (s, 1 H).

_N,N_-Dibenzyl-8-amino-3,6-dioxaoctanoic acid _t_-butyl ester (11)[16]

NaH (1.33 g of 95% wt/wt, 50 mmol) was added to a flask under argon with 100 mL DMF. Compound **10** (10 g, 35 mmol) was added and the mixture stirred at room temperature for 2 hrs. *t*-Butylbromoacetate was added to a dry flask under Ar at 0ºC. Solution from above was added via cannula and stirred at 0ºC for 30 min. The resulting solution was then allowed to warm to room temperature and stir overnight. 10 mL saturated NH_4Cl was added and the solution was concentrated to remove DMF. The remainder was partitioned between 100 mL sat. NH_4Cl and 150 mL EtOAc. The organic layer was washed with 100 mL NH_4Cl, 100 mL $NaHCO_3$ (1M), and 100 mL brine, then dried with $MgSO_4$ and concentrated to yield an amber oil. Purification on silica with 5:1 hexanes:EtOAc provided 9.5 g (68% yield) clear oil. ^{1}H-NMR ($CDCl_3$) δ 7.19-7.38 (m, 10 H), 3.94 (s, 1 H), 3.40-3.60 (m, 10 H), 2.69 (t, 2 H), 1.38 (s, 9 H).

8-amino-3,6-dioxaoctanoic acid _t_-butyl ester (12)

Compound **11** (6.4 g, 16 mmol) was dissolved in a solution of 750 μL AcOH in 150 mL MeOH. Palladium on charcoal (10% wt/wt) was added and the mixture was sealed in a Parr bomb and pressurized to ~7 atm H_2. The solution was stirred at room temperature for 18 hrs, then filtered through celite to remove Pd/C. The filtrate was concentrated to yield an amber oil, which was dissolved in 30 mL CH_2Cl_2 and washed

with 1M $NaHCO_3$ (20 mL) and concentrated. ^{1}H-NMR ($CDCl_3$) δ 4.24 (s, 2 H), 3.94 (t, 2 H), 3.71 (s, 4 H), 3.07 (t, 2 H), 1.45 (s, 9 H).

5α-dihydrotestosterone-*N*-(8-amino-3,6-dioxaoctanoic acid) carbamate (13)

12 (0.33 g, 1.5 mmol) and **15** (0.46 g, 1 mmol) were dissolved in 1 mL CH_3CN and DIEA (175 mL, 1 mmol) and stirred at 60°C for 24 hrs. The mixture was concentrated, suspended in 10 mL CH_2Cl_2 and washed with 2 x 20 mL 10% (wt/wt) citric acid and 20 mL water. The organic layer was dried over $MgSO_4$ and concentrated to yield a brown oil. This was treated with TFA (50% in CH_2Cl_2, 10 mL total volume, 2 hrs at room temperature) and concentrated to yield a brown oil. The crude product was run through a short column of silica with 2:1 hexanes:EtOAc, then eluted with MeOH and concentrated to an oily brown solid. Recrystallization from EtOH/water, resuspension in DCM, and concentration provided **13** as a light tan foam (0.154 g, 29% yield).

5α-dihydrotestosterone-4-nitrophenyl carbonate (15)

5α-dihydrotestosterone (2.0 g, 6.9 mmol) was dissolved in 40 mL CH_2Cl_2. *p*-nitrophenyl chloroformate (**14**) (1.7 g, 8.3 mmol) and triethylamine (1.15 mL, 8.3 mmol) were then added and the resulting solution was stirred at 35ºC for 12 hrs. The solution was concentrated, resuspended in 50 mL CH_2Cl_2, washed with 40 mL 1M $NaHCO_3$, 40 mL 10% (wt/wt) citric acid, and 40 mL brine. The organic portion was dried over $MgSO_4$ and concentrated to yield 2.83 g **15** (91% yield) as a fluffy white solid. ^{1}H-NMR ($CDCl_3$) δ 3.99 (s, 2 H), 3.65 (m, 4 H), 3.55 (t, 2 H), 3.33 (t, 2 H), 2.45-0.75 (series of m, 29 H), 1.47 (s, 9 H).

DHT-polyamides 4-9

13 (4.8 mg, 10 μmol) was activated as the –OBt ester with DCC (10 μmol) and HOBt (10 μmol) in DMF (60 μL) for 30 min at room temperatue. This was added to a solution of polyamide (each as **16**, possessing a free amine in the appropriate position and a *t*-Boc-protected (*R*) ^{H_2N}γ–turn, 2 μmol) and DIEA (1 μL, 6 μmol) in 50 μL DMF and the resulting reaction allowed to react for 1 hr at room temperature. Dp (2.5 μL) was added to quench remaining activated acid and the solution was concentrated to a brown oil in a speedvac. This was treated with 80% (v/v) TFA/CH_2Cl_2 for 2 hrs at room temperature, purified by C_{18} reverse-phase preparatory HPLC, and lyophilized to provide **4**-**9** as white powders. Characterization: **4**, MALDI-TOF $[M+H]^+$ (monoisotopic mass) calcd 1670.9, obsd 1671.0; **5**, MALDI-TOF $[M+H]^+$ (monoisotopic mass) calcd 1643.8, obsd 1643.9; **6**, MALDI-TOF $[M+H]^+$ (monoisotopic mass) calcd 1671.9, obsd 1672.0; **7**, MALDI-TOF $[M+H]^+$ (monoisotopic mass) calcd 1742.9, obsd 1743.1; **8**, MALDI-TOF $[M+H]^+$ (monoisotopic mass) calcd 1671.9, obsd 1672.0; **9**, MALDI-TOF $[M+H]^+$ (monoisotopic mass) calcd 1742.9, obsd 1743.0.

FITC-polyamides 17-19

Polyamides were synthesized on Kaiser oxime resin as previously detailed, each possessing a chiral (*R*) ^{H_2N}γ–turn protected as the *t*-butyl carbamate. Each polyamide (2 μmol) was dissolved in 100 μL DMF and 10 μL DIEA. Fluorescein isothiocyanate (3 μmol) was added and the reactions allowed to proceed in the dark at room temperature for 1 hr. The polyamides were precipitated by addition of Et_2O and treated with 100 μL 20% (v/v) TFA/CH_2Cl_2 for 1 hr at room temperature. The solutions were then

concentrated, suspended in 10 mL 0.1% (v/v) TFA/water, purified by C_{18} reverse-phase preparatory HPLC, and lyophilized to provide **17-19** as orange powders.

Gel Mobility Shift Assays

Gel shift assays were performed by Dr. Zhengxin Wang at the M.D. Anderson Cancer Center at the University of Texas Southwestern.

In vitro Transcription Reactions

In vitro transcription reactions were performed by Dr. Zhengxin Wang at the M.D. Anderson Cancer Center at the University of Texas Southwestern.

Transient Transfection Assays

Transient transfection assays were performed by Dr. Zhengxin Wang at the M.D. Anderson Cancer Center at the University of Texas Southwestern. Briefly, the human prostate cancer cell lines PC3 and LNCaP were cultured in a 5% CO_2 atmosphere at 37°C in RPMI medium 1640 supplemented with 10% fetal bovine serum (Irvine Scientific) and 1% penicillin/streptomycin solution (Sigma). The report vector was pGL3-ARE, containing 4 ARE's and the E4 core promoter fused to the luciferase gene. The expression vector is pcDNA-AR containing human AR under control of the CMV promoter. Assays were performed on 0.25 million cells, using 500 μg pcDNA-AR, 500 μg pGL3-ARE, 20 μg pRL-LUC as an internal control, and 10 μL LipofectAmine (Invitrogen). After 48 hrs, the cells were harvested for luciferase assay.

DNase I Footprinting Titrations

A 217 bp 5' ^{32}P-labeled PCR fragment was generated from template plasmid pPSA-ARE in accordance with standard protocols and isolated by nondenaturing gel electrophoresis.[17] All DNase I footprinting reactions were carried out in a volume of 400 μL. A polyamide stock solution or water (for reference lanes) was added to TKMC buffer, with final concentrations of 50 mM Tris-HCl, 50 mM KCl, 50 mM $MgCl_2$, and 25 mM $CaCl_2$, pH 7.0, and 15 kcpm 5'-radiolabeled DNA. The solutions were equilibrated for 12-18 h at 22°C. Cleavage was initiated by the addition of 10 μL of a DNase I stock solution and was allowed to proceed for 7 min at 22°C. The reactions were stopped by adding 50 μL of a solution containing 2.25 M NaCl, 150 mM EDTA, 0.6 mg/mL glycogen, and 30 μM base pair calf thymus DNA and then ethanol precipitated. The cleavage products were resuspended in 100 mM Trisborate-EDTA/80% formamide loading buffer, denatured at 85°C for 10 min, and immediately loaded onto an 8% denaturing polyacrylamide gel (5% cross-link, 7 M urea) at 2000 V for 2 h and 15 min. The gels were dried under vacuum at 80 °C and quantitated using storage phosphor technology.

References

1) Chang, C.; Kokontis, J.; Liao, S.T. *Science* **1988**, *240*, 324.

2) Chang, C.; Kokontis, J.; Liao, S.T. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 7211.

3) Chang, C.; Saltzman, A.; Yeh, S.; Young, W.; Keller, E.; Lee, H.-J.; Wang, C.; Mizokami, A. *Crit. Rev. Eukaryotic Gene Expression* **1995**, *5*, 97.

4) Evans, R.M. *Science* **1988**, *240*, 889 and references therein.

5) Truss, M.; Beato, M *Endocr. Rev*. **1993**, *14*, 459.

6) Cleutjens, K.B.J.M.; van Eekelen, C.C.E.M.; van der Korput, H.A.G.M.; Brinkmann, A.O.; Trapman, J. *J. Biol. Chem*. **1996**, *271*, 6379.

7) Luisi, B.F.; Xu, W.X.; Otwinowski, Z.; Freedman, L.P.; Yamamoto, K.R.; Sigler, P.B. *Nature* **1991**, *352*, 457.

8) Shaffer, P.L.; Jivan, A.; Dollins, D.E.; Claessens, F.; Gewirth, D.T. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4758.

9) Zhang, Y.L.; Wang, C.; Wilding, G.; Chang, C. *Biochem. Biophys. Res. Comm*. **1993**, *195*, 710.

10) Lee, H.-J.; Chang, C. Cell. Mol. Life Sci. 2003, 60, 1613.

11) Boffa, L.C.; Scarfi, S.; Mariani, M.R.; Damonte, G.; Allfrey, V.G.; Benatti, U.; Morris, P.L. *Cancer Res*. **2000**, *60*, 2258.

12) Sack, J.S.; Kish, .R.; Wang, C.; Attar, R.M.; Kiefer, S.E.; An, Y.; Wu, G.Y.; Scheffler, J.E.; Salvati, M.E.; Krystek, S.R., Jr.; Weinmann, R.; Einspahr, H.M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4904.

13) Baird, E.E.; Dervan, P.B. *J. Am. Chem. Soc*. **1996**, *118*, 6141.

14) Belitsky, J.M.; Nguyen, D.H.; Wurtz, N.R.; Dervan, P.B. *Bioorg. Med. Chem*. **2002**, *10*, 2767.

15) Koskinen, A.M.P.; Valo, T.; Vihavainen, S.; Hakala, J.M.L. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 573.

16) Wittmann, V.; Takayama, S.; Gong, K.W.; Weitz-Schmidt, G.; Wong, C.-H. *J. Org. Chem*. **1998**, *63*, 5137.

17) Trauger, J.W.; Dervan, P.B. *Methods Enzymol*. **2001**, *340*, 450.