Chapter 5

Suppression of Androgen Receptor Mediated Gene Expression by a Sequence-Specific DNA Binding Polyamide

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Abstract

Androgen Receptor (AR) is essential for the growth and progression of prostate cancer in both hormone-sensitive and hormone-refractory disease. A DNA binding polyamide that targets the consensus androgen response element (ARE) binds the PSA promoter ARE, inhibits androgen-induced expression of PSA and several other AR-regulated genes in cultured prostate cancer cells, and reduces AR occupancy at the PSA promoter and enhancer. Down-regulation of PSA by this polyamide was comparable to that produced by the synthetic anti-androgen bicalutamide (Casodex) at the same concentration. Genome-wide expression analysis reveals that a similar number of transcripts are affected by treatment with the polyamide and with bicalutamide. Direct inhibition of the AR-DNA interface by sequence-specific DNA binding small molecules could offer an alternative approach to antagonizing AR activity.

5.1 Introduction

The androgen receptor (AR) is a member of the ligand-activated nuclear receptor family of transcription factors with a conserved DNA binding domain containing contains two modules of zinc coordinated by four cysteines (Figure 5.1) (1). Ligand binding to AR initiates release from the cytoplasm, dimerization, binding to the androgen response elements (ARE) of target genes, and gene activation through interaction with co-activators and the general transcription machinery (2). Functional AREs, consensus sequence 5'-GGTACAnnnTGTTCT-3' (Figure 5.3A) (3), can occur in proximal promoter sequences or in enhancers located up to several thousand base pairs upstream or downstream of the transcription start site.

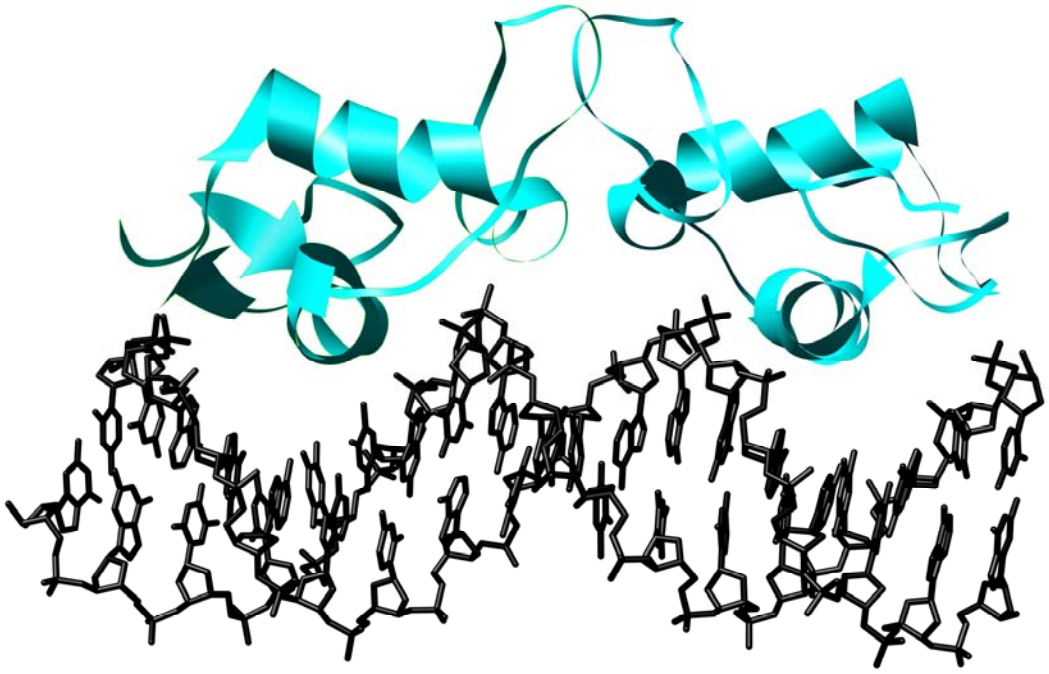

Figure 5.1 Crystal structure of androgen receptor homodimer bound to the sequence 5'-AGAACATCAAGAACAG-3' (34).

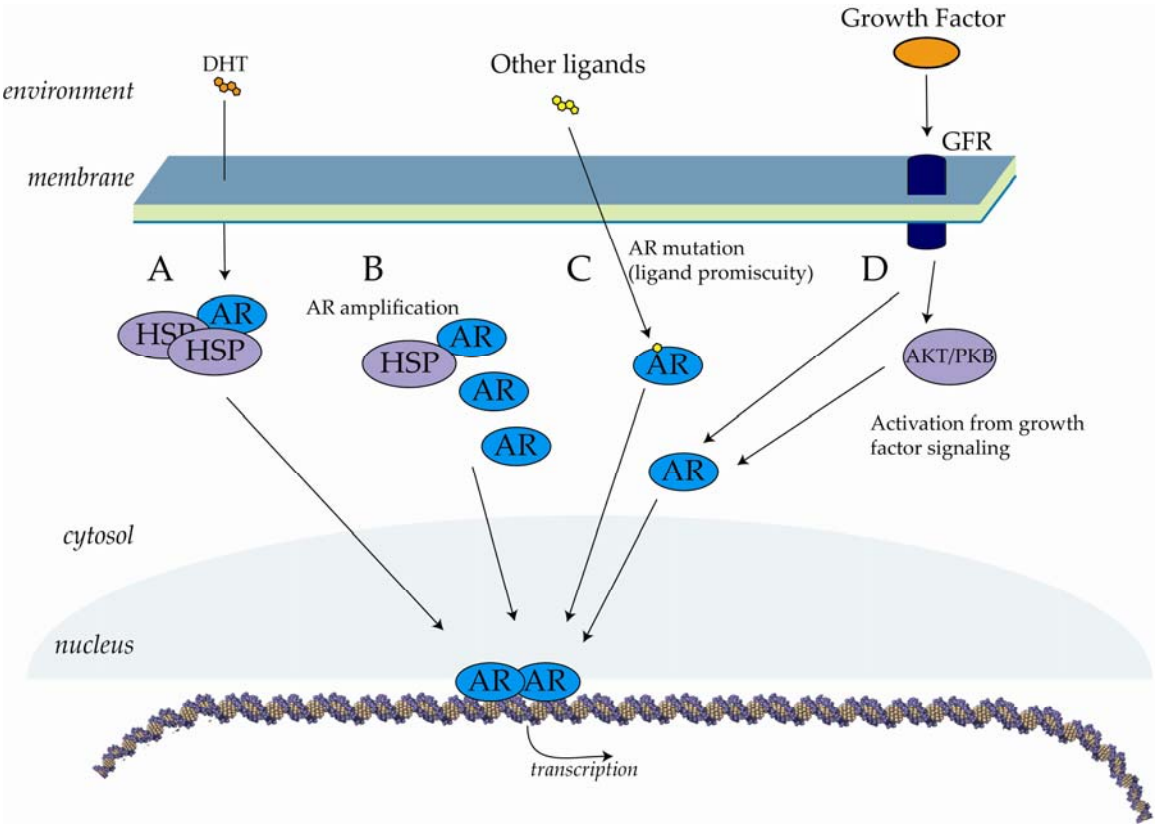

Figure 5.2 Androgen receptor activation in androgen independent prostate cancer (A) Normal activation of AR by DHT. (B) AR amplification leads to activation by mass action. (C) Mutations in AR leads to promiscuity for other ligands. (D) Ligand-independent activation by upstream signaling.

The regulation of prostate specific antigen (PSA, KLK3) expression by AR has been extensively studied as a model for AR mediated gene activation (4-7). Androgenic induction of PSA is mediated by AR binding to the proximal promoter approximately 170 base pairs from the transcription start site, and to several low-affinity AREs in an enhancer approximately 4000 base pairs upstream (4-6). AREs in both the promoter and enhancer are important for induction after androgen stimulation. AR occupies both the promoter and enhancer regions and recruits transcriptional coactivators including p160 and p300, TATA binding protein, mediator, and RNA polymerase II to form the AR

transcription complex (7, 8). Chromatin capture assays suggest that the PSA enhancer is located near the promoter in this complex (8).

AR signaling regulates normal prostate development and contributes to the progression of prostate cancer (9). Surgical or drug therapies that act to limit circulating androgen levels or directly antagonize ligand binding to AR initially slow prostate cancer growth (10, 11). However, nearly all patients treated with anti-androgen therapies will eventually develop hormone-refractory disease (12). Dysregulation of AR activity (Figure 5.2), together with activation of the PTEN/AKT pathway, is thought to contribute to this transition (13). Up-regulation of AR mRNA was found to occur in all transitions from hormone-sensitive to hormone-refractory disease in a mouse tumor-xenograft model of prostate cancer (14). Additionally, a transgenic mouse with a mutated AR that inappropriately interacts with transcriptional co-regulators developed metastatic neoplastic disease (15). Mutations in the AR ligand binding domain can render antagonists such as bicalutamide or flutamide ineffective, or in some models of hormone-refractory disease convert them to agonists (14, 16). Since genotropic AR activity is thought to be necessary throughout prostate cancer progression, direct antagonism of AR:DNA binding could inhibit androgen receptor activity in hormone-refractory conditions where androgen antagonists that target the ligand-binding pocket are ineffective (9).

DNA binding polyamides represent one approach to inhibiting protein-DNA interactions. Polyamides containing *N*-methylimidazole (Im) and *N*-methylpyrrole (Py) comprise a class of programmable DNA-binding ligands capable of binding to a broad repertoire of DNA sequences with affinities and specificities comparable to those of

natural DNA-binding proteins (17, 18). Sequence specificity is programmed by side-by-side pairings of the heterocyclic amino acids in the minor groove of DNA: Im/Py distinguishes G•C from C•G; Py/Py binds both A•T and T•A (19, 20). Previously, a hairpin polyamide targeted to the hypoxia response element (HRE) inhibited hypoxia induced expression of several HIF-1 regulated genes including VEGF in cultured cells (21, 22).

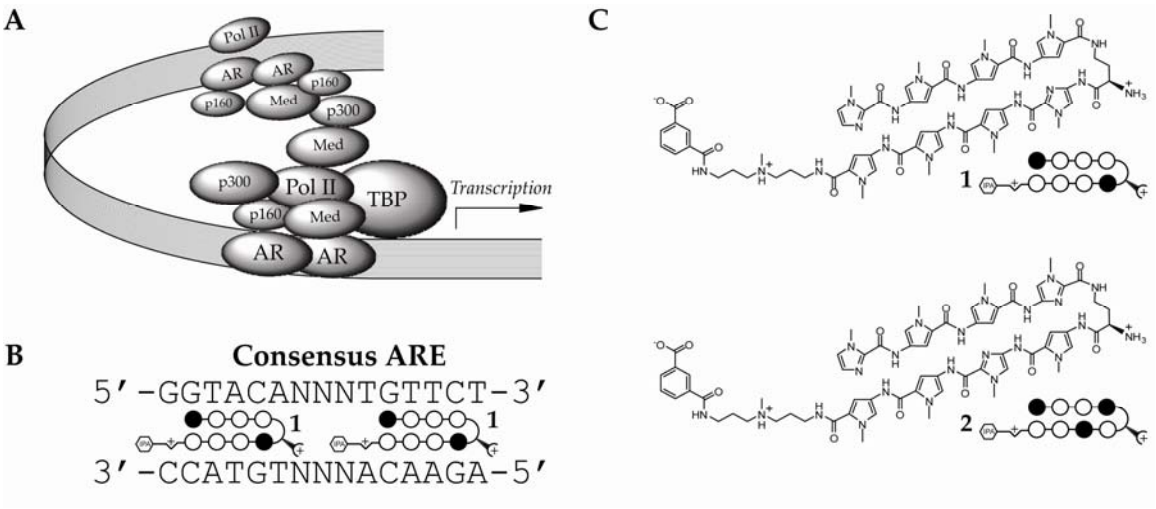

Figure 5.3 (A) Model of the androgen receptor (AR) transcription complex. (B) Consensus androgen response element. (C) Structures and ball-and-stick models of polyamide **1**, designed to bind the consensus ARE, and **2**, a two base-pair mismatch. Imidazole and pyrrole monomer units are represented by closed and open circles, respectively. The isophthalic acid tail moiety is represented by a hexagon.

In this study we have designed a cell permeable polyamide to target the sequence 5'-WGWWCW-3', found in the consensus ARE, with the goal of disrupting AR mediated gene expression (Figure 5.3). We show that this polyamide binds the ARE found in the PSA promoter, inhibits expression of PSA as well as approximately 35% of the transcripts that were induced by dihydrotestosterone (DHT) in cultured prostate

cancer cells, and reduces AR occupancy at the PSA promoter and enhancer. Down-regulation of PSA by this polyamide was comparable to the effects of the synthetic anti-androgen bicalutamide (Casodex) at the same concentration. A control polyamide targeted to a different sequence had much less effect.

5.2 Results

Binding affinities of polyamides to the ARE of the PSA promoter. The proximal PSA promoter contains the ARE 5'-AGAACAGCAAGTGCT-3' (Figure 5.4A). The DNA-binding of polyamides **1** and **2** on this sequence was measured by quantitative DNase I footprint titrations using a 5'-^{32}P-labeled PCR fragment of pAR-PSA which contains the PSA ARE. Polyamide **1** has a $K_a = 8.3 \pm 1.7 \times 10^9\ M^{-1}$ for the ARE consensus half site 5'-AGAACA-3' (Figure 5.4B). Binding of polyamide **2**, which targets the sequence 5'WGWCGW-3', to the ARE is not measurable by these methods ($K_a < 1 \times 10^7$) (Figure 5.4C). Minimal binding of polyamide **1** is observed at the other half site of the ARE: 5'-AGTGCT-3', which is formally a single basepair mismatch site for **1**. However, **1** is observed to bind the sequence 5'-AGATCA-3' approximately 12 base pairs 5' to the ARE, which is an expected binding site for this molecule.

Electrophoretic Mobility Shift Assay. The effects of polyamides **1** and **2** on the binding of factors present in the nuclear extract isolated from DHT-stimulated LNCaP cells to the ARE site in the PSA promoter was measured by an electrophoretic mobility shift assay (Figure 5.4D). Polyamide **1** inhibits binding to the 5'-^{32}P labeled duplex at concentrations as low as 10 nM. Polyamide **2** has minimal effect at the same concentrations.

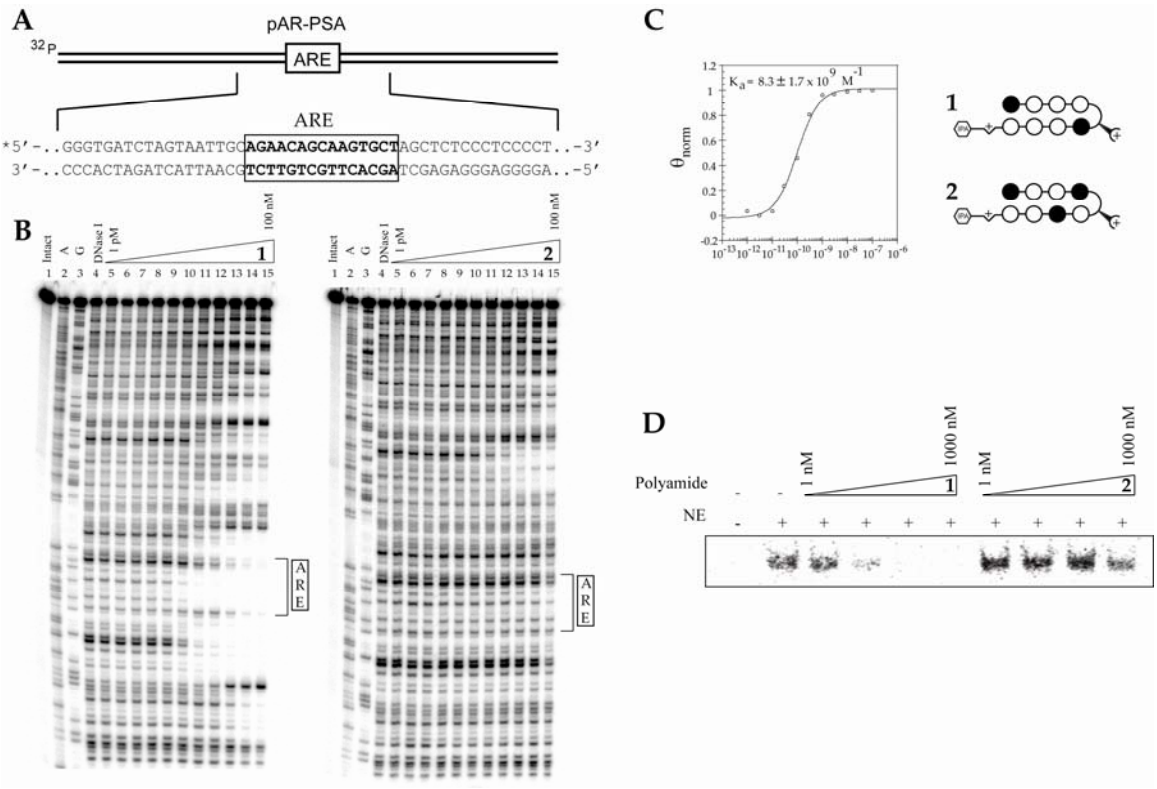

Figure 5.4 Binding of **1** and **2** to the ARE in the PSA promoter. (A) Illustration of pAR and partial sequence of the PSA promoter. (B) Quantitative DNase I footprint titration experiments for polyamides **1** and **2** on the 5'-end-labeled PCR product of plasmid pAR-PSA: lane 1, intact DNA; lane 2, A reaction; lane 3, G reaction; lane 4, DNAse I standard; lanes 5–15, 1 pM, 3 pM, 10 pM, 30 pM, 100 pM, 300 pM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM polyamide, respectively. (C) Isotherm for **1** binding to the ARE half site 5'-AGAACA-3'. Polyamide **1** has a $K_a = 8.3 \pm 1.7 \times 10^9$ for this site. Polyamide **2** shows no measurable binding in the footprinted region. (D) EMSA of DHT-stimulated LNCaP cell nuclear extract (NE) binding to a 31 base pair oligonucleotide duplex containing the PSA promoter ARE in the presence of **1** and **2**.

Inhibition of androgen-inducted PSA expression. Induction of PSA mRNA by DHT in the presence of polyamides **1**, **2**, and bicalutamide in LNCaP cells was measured by quantitative real-time RT–PCR. Bicalutamide and polyamide **1** inhibit the expression of DHT-induced PSA in a dose dependent manner up to approximately 70% at 10 μM as measured in this assay (Figure 5.5A). Polyamide **2** has a more modest effect. Secretion of PSA protein after DHT stimulation of LNCaP cells in the presence of **1** and **2** was

measured by ELISA (Figure 5.5B). Supernatant concentrations of PSA protein are reduced in cells pretreated with **1** as compared to **2** or an untreated control. AR occupancy at the PSA promoter and enhancer was assessed by chromatin immunoprecipitation (Figure 5.5C). Chromatin immunoprecipitation assays with anti-AR antibody treatment indicate decreased occupancy of AR at the PSA promoter and enhancer in the presence of 10 μM **1**. Polyamide **2** has minimal effect. Polyamides **1** and **2** display no obvious detrimental effects on cell growth over the course of the experiment.

Inhibition of androgen-induced FKBP5 expression. Recent studies have identified FKBP5 as one of the most strongly induced genes in androgen stimulated prostate cancer cells (23). Two functional AREs with the sequences 5'-AGCACATCGAGTTCA-3' and 5'-AGAACAGGGTGTTCT-3' have been mapped to an enhancer within the fifth intron (24). Polyamide **1** inhibits DHT-induced expression of FKBP5 by approximately 60% (Figure 5.5D). Bicalutamide was more potent, however, inhibiting expression by almost 95%. Polyamide **2** has minimal affect on FKBP5 expression. Chromatin immunoprecipitation assays indicate decreased occupancy of AR at the FKBP5 intronic enhancer in the presence of 10 μM **1** (Figure 5.5E), whereas polyamide **2** has no measurable effect.

Global effects on androgen-induced gene expression. Global effects of polyamides **1**, **2**, and bicalutamide on gene expression in DHT stimulated LNCaP cells were monitored using Affymetrix high-density Human Genome U133 Plus 2.0 Arrays which interrogate over 50,000 transcripts. As compared to DHT-induced controls, polyamide **1** (10 μM) affected the expression of 1,053 transcripts by at least 2-fold ($p \leq 0.01$) (Table 5.1), which represents less than 2% of interrogated transcripts. Of this total, 706 were down-

regulated. At the same threshold, bicalutamide (10 μM) affected the expression of 1,213, with 602 of those down-regulated. Polyamide **2** (10 μM) affected the expression of 379 transcripts, which represents less than 1% of interrogated transcripts. A divisive clustering analysis over all interrogated transcripts suggests that the expression profiles of cells treated with bicalutamide, **1**, and **2** are largely distinct (Figure 5.6A). Analysis of transcripts affected by both bicalutamide and **1** shows that 122 and 90 transcripts are commonly down- and up- regulated, respectively, at least 2-fold ($p \leq 0.01$) (Figure 5.6B). Of the 122 transcripts down-regulated by both bicalutamide and **1**, 117 are also observed to be induced by DHT at the same thresholds. Of the 90 up-regulated transcripts, 59 are observed to be repressed by DHT.

The response of cultured prostate cancer cells to androgen has been extensively studied (23, 25). We find that DHT induced the expression of a set of 199 transcripts by at least 4-fold ($p \leq 0.01$) (Appendix B, Table B1). Of this set, 70 were also inhibited by polyamide **1** by at least 2-fold ($p \leq 0.01$) (Appendix B, Table B2). For comparison, polyamide **2** inhibited 20, and bicalutamide inhibited 186 of the 199 DHT induced transcripts using the same thresholds (Figure 5.6C). We find DHT repressed the expression of a set of 88 transcripts by at least 4-fold ($p \leq 0.01$). Of this set 8 were also de-repressed, as compared to DHT-treated controls, by polyamide **1** by at least 2-fold ($p \leq 0.01$). For comparison, polyamide **2** de-repressed 3, and bicalutamide de-repressed 87 of the 88 transcripts repressed by DHT using the same thresholds (Figure 5.6C). A complete list of the DHT-induced transcripts and those affected by **1** is provided in an appendix of this thesis. It is not known what proportions of these genes are direct targets of AR. Table 5.2 displays the effects of each treatment on the expression of a few

selected genes that were observed to be induced by DHT and are known to be targets of AR (26, 27). Effects on the expression of KLK2 and TMPRSS2 were verified by quantitative real-time RT–PCR (Figure 5.7).

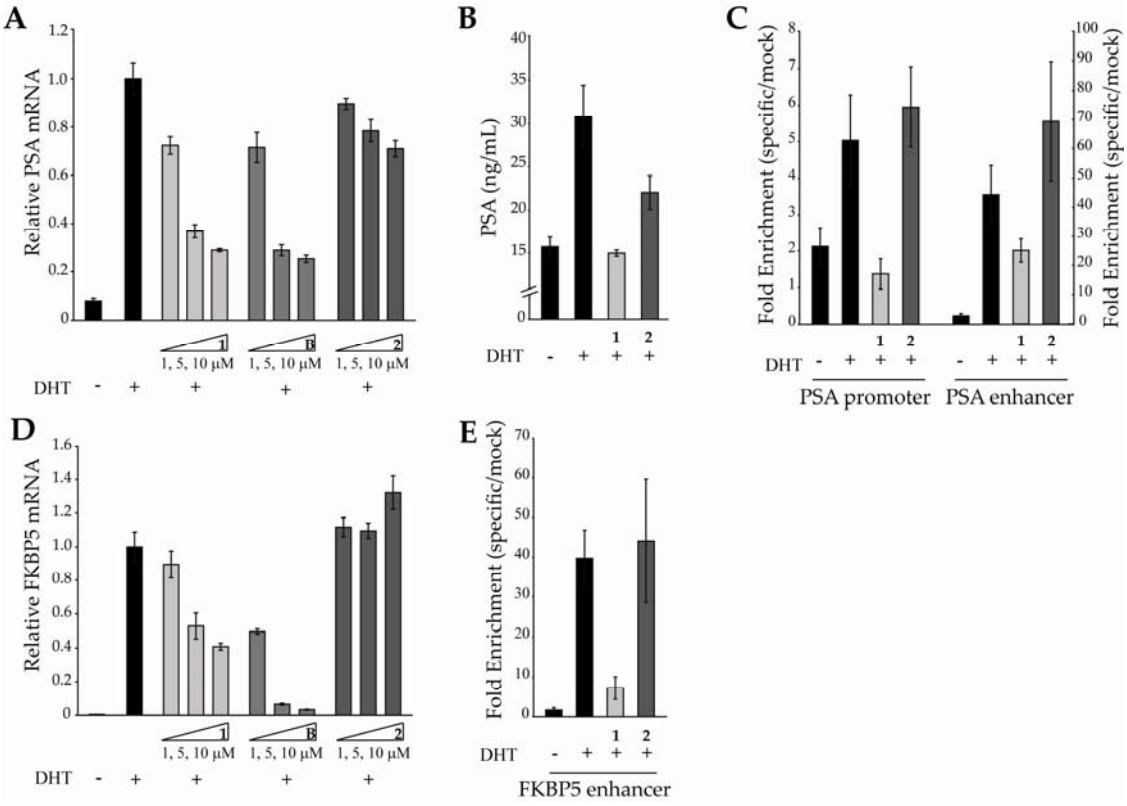

Figure 5.5 Inhibition of DHT-induced PSA and FKBP5 expression by **1** and **2**. (A) Induction of PSA mRNA in the presence of **1**, **2**, and bicalutamide, B, measured by quantitative real-time PCR. **1** and bicalutamide inhibit expression of PSA in a dose-dependent manner up to approximately 70% at 10 μM. **2** has a more modest effect. (B) Secreted PSA protein measured by ELISA. (C) Chromatin immunoprecipitation assays with anti-AR or mock antibody treatment expressed as fold-enrichment (specific/mock) of DNA sequences at the PSA promoter and enhancer. AR occupancy at the PSA promoter and enhancer is decreased in the presence of **1** (10 μM) but not **2**. (D) Induction of FKBP5 mRNA in the presence of **1**, **2**, and bicalutamide, **B**. (E) Chromatin immunoprecipitation assays with anti-AR at the FKBP5 fifth intron enhancer. Polyamide concentrations are 10 μM.

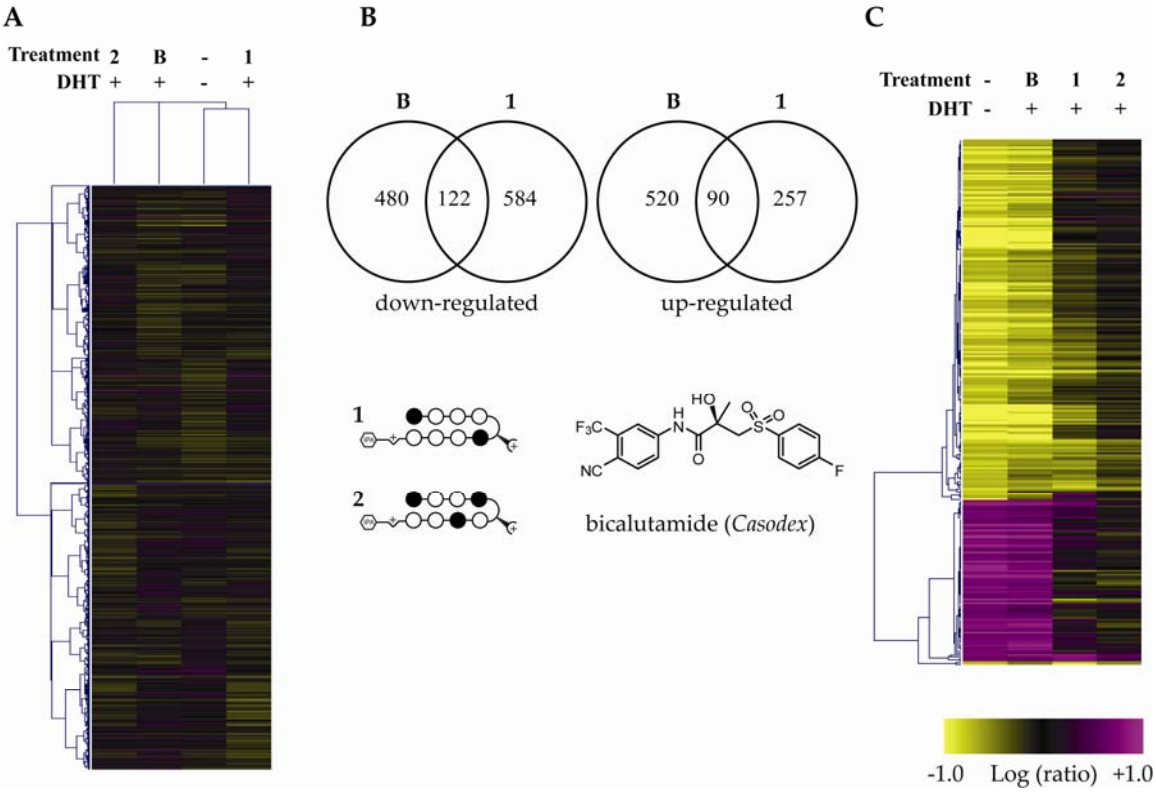

Figure 5.6 Global effects on transcripts interrogated using Affymetrix high-density Human Genome U133 Plus 2.0 Arrays. (A) Divisive clustering of all measured transcripts under the four specified conditions: no treatment control; **B**, bicalutamide (10 μM); **1** (10 μM); **2** (10 μM). Clustering was based on an error weighted Pearson correlation of intensity ratios for each treatment as compared to DHT-induced controls. (B) Ven diagrams representing transcripts down- and up-regulated ($|\text{fold-change}| \geq 2.0$, $p \leq 0.01$) by bicalutamide and **1**. Numbers inside the intersections represent transcripts affected by both treatments. Of the 122 transcripts down-regulated by both bicalutamide and **1**, 117 are also observed to be induced by DHT at the same thresholds. (C) Agglomerative clustering of expression changes of the 199 transcripts induced or repressed 4-fold ($p \leq 0.01$) or more by 1 nM DHT under the designated treatment conditions. Of the DHT-induced set, 70 were inhibited by polyamide **1**, 20 were inhibited by **2,** and 186 by bicalutamide ($|\text{fold-change}| \geq 2.0$, $p \leq 0.01$). Clustering parameters were the same as in (A). Treatments reported are an error-weighted average from three experiments, except the non-induced control which was an average from two experiments.

Treatment	-	B	1	2
DHT	-	+	+	+
up-regulated (fold change ≥ 2.0)	486	611	347	95
down-regulated (fold change ≤ -2.0)	782	602	706	284
up-regulatd (fold change ≥ 4.0)	88	96	42	11
down-regulated (fold change ≤ -4.0)	199	133	126	32

Table 5.1 Number of transcripts affected relative to DHT-induced controls. ($p \leq 0.01$)

Treatment	-	B	1	2
Gene DHT	-	+	+	+
KLK2	-23.0	-14.7	-2.4	-1.1
KLK3 (PSA)	-6.1	-3.2	-3.3	-1.4
TMPRSS2	-6.2	-4.1	-2.3	-1.4
FKBP5	-42.9	-36.4	-3.1	1.5

Table 5.2 Fold-changes of selected AR-target genes relative to DHT-induced controls.

5.3 Discussion

Because numerous signaling pathways converge on a smaller number of transcription factors to exert their effects on gene expression, it has been proposed that transcription factors could be among the most appropriate drug targets in oncology (28, 29). This has underscored the challenge to design small molecules capable of selectively disrupting protein-protein interactions between co-activators as well as protein-DNA interactions between transcription factors and their target sites in gene regulatory sequences.

Prostate cancer cells are dependent on stimulation by circulating androgens that exert their effects through the androgen receptor signaling axis. Hormone therapies that block AR activity by starving it of androgens or inhibiting ligand binding are initially successful but ultimately fail to control disease (12). This can occur through up-

regulation of AR, mutations in the ligand binding pocket, and ligand-independent activation from upstream signaling proteins (13, 30, 31). It is thought, however, that intact activity of AR signaling is necessary for disease progression (9). Inhibition of the AR-DNA interaction by a sequence-specific DNA binding molecule could be expected to interfere with AR signaling under both hormone-sensitive and refractory conditions.

Polyamide **1** binds to a half-site of the ARE of the PSA promoter with a subnanomolar K_D and inhibits expression of approximately 35% of transcripts that are observed to be induced 4-fold or more by DHT in LNCaP cells. Down-regulation of PSA by this polyamide is comparable to that produced by the synthetic anti-androgen bicalutamide at the same concentration. Control polyamide **2**, which targets a different DNA sequence, 5-WGWCGW-3', had significantly less effect on androgen induced gene expression. Expression of PSA (KLK3), KLK2, TMPRSS2, and FKBP5, which are direct AR targets, were all affected by **1**. TMPRSS2 encodes a transmembrane protease and can undergo a chromosomal deletion in which a member of the ETS transcription factor family is placed under control of the strongly androgen responsive TMPRSS2 5' regulatory region (27, 32).

At the same concentration, polyamide **1** and bicalutamide affected a comparable number of transcripts, while polyamide **2** affected significantly fewer. Using bicalutamide as a point of reference, the overall effects on genomic transcription by **1** and **2** are relatively modest. Although it is difficult to compare across experimental conditions, the observation that a limited number of genes are affected by each polyamide in this study is consistent with previous reports (21). A comparison of the expression data for cells treated with polyamide **1** or **2** reveal that some transcripts are similarly

affected, but many are differentially affected by the two polyamides (Figure 4A), which is consistent with previous comparisons of gene expression profiles of cells treated with polyamides of different target sequence (21) (33).

The androgen receptor, glucocorticoid receptor, and estrogen receptor share a highly conserved DNA binding domain (34-36). This domain, related to the classical Cys-2-His-2 zinc finger motifs (37), contains two modules of zinc coordinated by four cysteines. Previously, a polyamide targeted to the estrogen receptor response element inhibited binding of estrogen receptors alpha and beta in gel-shift assays (38). In separate *in vitro* experiments, minor groove binding polyamides have been shown to inhibit the major groove binding of Zif268 and other zinc finger proteins to their target sites on DNA by an allosteric mechanism (39). In light of this, it is not unexpected that a polyamide targeted to the ARE would inhibit AR binding.

The ARE is sufficiently degenerate such that a single polyamide is not likely to affect all AR-regulated genes simultaneously. The identities of the particular AR target genes involved in prostate cancer progression are not fully known. In the absence of this knowledge, it was our goal to target the ARE broadly to maximize the number of AR target genes affected using a single polyamide. However, the programmability of polyamides might allow selective inhibition of a predetermined subset of AR target genes by one or a small cocktail of tailored polyamide molecules. The utility of disrupting the AR-ARE interface with DNA binding small molecules will depend on continued experimentation in small animal models of hormone refractory prostate cancer and AR regulated gene expression (40-42).

5.4 Methods

Synthesis of Polyamides. Polyamides **1** and **2** were synthesized by solid-phase methods on Kaiser oxime resin (Nova Biochem) according to established protocols (43). Polyamides were cleaved from resin with 3,3'-diamino-*N*-methyl-dipropylamine and purified by reverse-phase HPLC. Isophthalic acid was activated with PyBOP (Nova Biochem) and conjugated to the polyamides as previously described (22). Purities and identities of the polyamides were assessed by HPLC, UV-visible spectroscopy, and MALDI-ToF MS.

Determination of DNA-binding affinity and sequence specificity. Plasmid pAR-PSA was constructed by inserting a seventy base pair sequence from the PSA promoter containing the ARE into pUC19 plasmid. Quantitative DNase I footprint titration experiments were used to measure the binding affinities of **1** and **2** on a 5'- ^{32}P labeled fragment of pAR-PSA that contains the PSA promoter ARE. Detailed experimental protocols are reported elsewhere (44).

Electrophoretic Mobility Shift Assay. The oligonucleotide 5'-GCATTGCAGAACA-GCAAGTGCTAGCTCTCCC-3' containing the PSA promoter ARE (underscored) was end labeled with ^{32}P and annealed to its complement. Polyamides **1** and **2** were incubated with the duplex for three hours in previously optimized buffer conditions (45). Nuclear extract from DHT treated LNCaP cells (Genetex) was then added for an additional 45 minutes. Complexes were run on a 5% polyacrylamide gel and visualized on a phosphorimager.

Measurement of Androgen-Induced PSA mRNA and Protein. LNCaP cells (ATCC) were plated in 24-well plates at a density of 40-50 x 10^3 cells per well (80-100 x 10^3

cells/ml) in RPMI (ATCC) supplemented with 10% FBS (Irvine Scientific). After 72 hours, the medium was replaced with RPMI containing 10% charcoal stripped FBS with or without polyamides at the designated concentrations. Cells were grown for an additional 48 hours and then treated with 1 nM dihydrotestosterone (DHT) for 16 hours. When appropriate, bicalutamide was added two hours prior to DHT stimulation. Isolation of RNA and cDNA synthesis were as previously described (21). Quantitative real-time RT–PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7300 instrument. PSA mRNA was measured relative to ß-glucuronidase as an endogenous control. Primer sequences are available upon request. Cell culture supernatants were collected for an ELISA (R&D Systems) to measure PSA protein according to the manufacturer's protocol.

Chromatin Immunoprecipitation. LNCaP cells were plated in 15 cm diameter plates at a density of 2 x 10^6 cells per plate. Media, polyamide treatment, time course, and DHT stimulation were the same as described above. After the 16 hour DHT treatment, cells were treated with 1% formaldehyde for 10 minutes. Chromatin was isolated and sheared. Antibodies to AR (AR-20, Santa Cruz Biotechnology) were used to immunoprecipitate AR-bound DNA fragments. Crosslinks were reversed and PCRs using primers targeted to the regions of interest were used to assess enrichment of bound fragments as compared to mock-precipitated (no antibody) controls. PCRs were monitored using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7300 instrument. Primer sequences and a more detailed experimental protocol are available upon request. See Appendix C for a more detailed protocol.

Analysis of Gene Expression with Oligonucleotide Microarrays. LNCAP cells were plated in 12-well plates at a density of 80-100 x 10^3 cells per well. Media, polyamide treatments, and time courses were the same as described above. Two hours prior to DHT stimulation, bicalutamide was added. RNA was isolated as previously described. From this point, experiments were carried out at the Millard and Muriel Jacobs Gene Expression Facility at the California Institute of Technology. Labeled mRNA was hybridized to Affymetrix high-density Human Genome U133 Plus 2.0 Arrays according to established protocols. Gene expression was analyzed using Resolver (Rosetta Biosoftware, Seattle). Data was uploaded to the Gene Expression Omnibus repository (GSE7708).

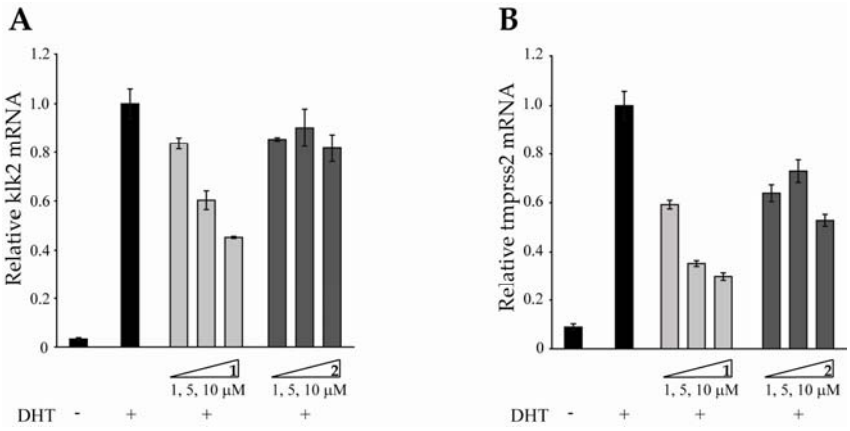

Figure 5.7 DHT-induction of KLK2 mRNA (A) and TMPRSS2 mRNA (B) in the presence of **1**, **2**, and bicalutamide, **B**, measured by quantitative real-time PCR.

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