

Chapter 4

Characterization of Py-Im Polyamide Androgen Receptor Antagonists in Hormone-Refractory Prostate Cancer Cells

John W. Phillips and Peter B. Dervan

Abstract

We have previously developed DNA-binding polyamides targeted to the consensus androgen response element (ARE). These compounds bind to DNA in the nucleus of live, hormone-sensitive cultured prostate cancer cells and reduce the occupancy of androgen receptor at some AREs, including the one in the intronic enhancer region of FKBP5. Expression of FKBP5, KLK3 and other androgen receptor (AR) target genes is correspondingly reduced. In this study, we investigate the activity of these compounds in the LNCaP-AR cell line, which is a tissue culture model of hormone-refractory disease. We determine that the polyamides maintain their activity against PSA mRNA expression but fail to decrease AR occupancy at the FKBP5 ARE in LNCaP-AR cells, which suggests an alternative mechanism for inhibition of AR-target gene expression exists in this cell line. Cytotoxic stress is also known to disrupt AR signaling; we show that p53 is stabilized and programmed cell death pathways are activated in response to polyamide treatment. Further investigation into the cytotoxic effects of polyamide treatment is recommended.

4.1. Introduction

The dramatic clinical response of primary prostate cancer to androgen deprivation therapy highlights the importance of the androgen receptor (AR) in its pathogenesis (1). In fact, chemical or surgical castration can establish clinical remission in many patients. Pharmacologic agents used for this purpose include bicalutamide, a synthetic anti-androgen that binds the ligand-binding pocket of the AR and prevents assembly of the coactivators required for AR-driven gene expression (2,3). For many patients, bicalutamide and other hormonally active agents maintain efficacy at least six months (4). When the disease recurs, it is invariably resistant to conventional hormonal therapy, metastatic, and lethal.

Recent studies have shown that the progression to hormone refractory prostate cancer (HRPC) is critically dependent on androgen receptor signaling. Recurrent tumors often show evidence of AR function despite low levels of circulating androgens (5). Mechanisms proposed to explain the reactivation of AR signaling include mutation of the ligand binding domain or upregulation of the receptor, ligand-independent AR stimulation through other signaling pathways, and changes in AR-coactivators (4). Of all of these, gene amplification is likely the most important mechanism of resistance, as it is the expression change most often found in patient samples of recurrent disease and the only change common to several mouse xenograft models of HPRC. AR remains a crucial therapeutic target even after first-line anti-hormone therapies have failed.

We have previously reported the characterization of a Py-Im polyamide antagonist that can disrupt AR binding to androgen response elements (ARE) in the hormone-sensitive prostate cancer cell line LNCaP (6). Polyamides are programmable, high-affinity, DNA sequence-specific molecules that can permeate cells, traffic to the nucleus, and bind DNA in chromatin (7,8). The polyamide androgen antagonist was designed to bind 5'-WGWWCW-3' (W = A or T) sequences in DNA, which blocked AR binding at a subset of the 5'-GGTACAnnnTGTTCT-3' sequences in regulatory regions of AR-target genes

(Figure 4.1a). This small molecule inhibited the dihydrotestosterone (DHT)-induced expression of many AR-target genes, including *TMPRSS2*, *FKBP5*, and *PSA*. The activity of the polyamide was comparable to bicalutamide, a synthetic anti-androgen currently in clinical use.

Given the dependence of recurrent prostate cancer progression on AR and the ability of a simple upregulation of AR expression to defeat anti-androgens targeted to the ligand-binding pocket, the continued ability to abrogate AR signaling in recurrent prostate cancer disease models by disrupting the protein-DNA interface would represent an important proof of concept. In this study, we explore the activity of a polyamide-ARE antagonist in a cell culture model of recurrent, hormone-sensitive prostate cancer: the LN-AR cell line (5). This cell line is a derivative of LNCaP cells containing a stably integrated vector that overexpresses

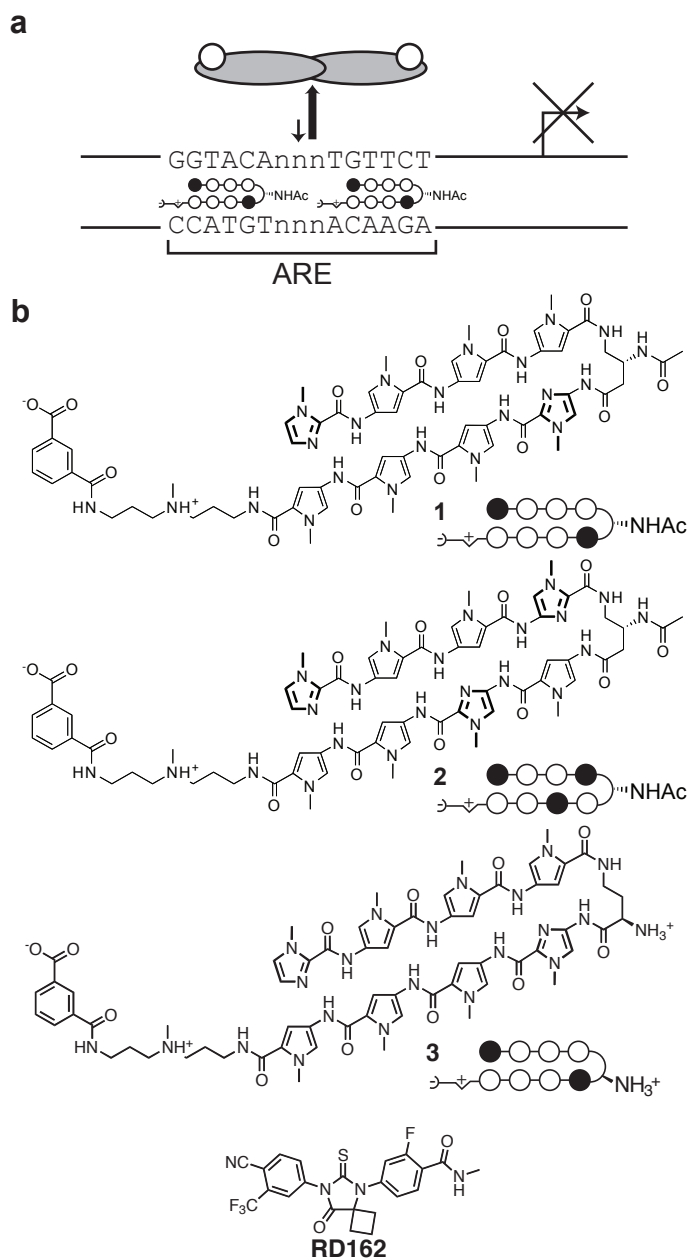


Figure 4.1. Disrupting the AR/ARE interface in HRPC. (a) A DNA-binding polyamide targeting the ARE consensus sequence disrupts the AR/ARE interface and offers an alternative anti-androgen strategy. (b) Chemical structures and ball-and-stick representations of the polyamides and the synthetic anti-androgen RD162.

AR. In this model, traditional anti-androgens like bicalutamide lose their efficacy. In fact, AR overexpression *in vitro* transforms bicalutamide into an agonist of AR activity. It is possible that compounds targeted to the protein-DNA interface would not be defeated by this resistance mechanism.

In this study we investigate the viability of targeting the protein-DNA interface as an alternative anti-androgen strategy for recurrent prostate cancer. We examine the activity of an improved polyamide ARE antagonist (compound **1**) in LNCaP and LN-AR cells (Figure 4.1b). We compare its activity to that of the original polyamide ARE antagonist (compound **3**). These studies also include mismatch compound **2**, which is targeted to the unrelated DNA sequence 5'-WGWCGW-3'. Additionally, we include a comparison of the polyamides with the second- and third-generation anti-androgens, bicalutamide and RD162 (9). We show that **1** maintains its potency against AR-driven mRNA expression in LN-AR cells, but that it no longer blocks AR binding to the ARE of a known AR-responsive gene, FKBP5. We show that the inhibition of PSA mRNA expression produced by **1** is accompanied by activation of cell stress and programmed cell death pathways. These results have important implications for the proposed mechanism of AR inhibition by polyamides.

4.2. Results

4.2.1. Compound synthesis. Using a combination of solution- and solid-phase techniques, we prepared an improved ARE-antagonist polyamide (compound **1**) that incorporated an acetylated beta-amino hairpin turn (Figure 4.1). This design change was recently demonstrated to improve the affinity and biological activity of the alpha-amino compound used in our hormone-sensitive studies. We also synthesized an appropriate mismatch compound designed to bind an unrelated sequence (5'-WGWCGW-3') (compound **2**). Compound **3** is the original, unacetylated alpha-turn compound from the hormone sensitive cell lines studies and is included in some assays as a control. We also synthesized the third-generation anti-androgen, RD162, using previously-published protocols for use as an additional control (9).

4.2.2. Inhibition of PSA expression in LNCaP and LN-AR cells. We tested the ability of the improved ARE-match polyamide **1** to inhibit AR-driven gene expression in LNCaP and LN-AR cells and compared its activity to the mismatch compound **2** and to bicalutamide. Using qPCR analysis of DHT-induced PSA mRNA expression, we found that polyamide **1** inhibited AR-driven gene expression in the hormone-sensitive and the hormone-refractory setting (Figure 4.2a, b). The polyamide also inhibited basal PSA expression in hormone-stripped media without DHT; bicalutamide acted as an agonist in this setting (Figure 4.2c). The inhibition was dose dependent and efficacious, as PSA levels could be reduced to their non-induced levels. The IC_{50} for the inhibition of PSA mRNA by **1** was approximately $0.5 \mu\text{M}$ in LNCaP cells and $0.3 \mu\text{M}$ in LN-AR cells (Table 4.1). The downregulation was also sequence specific, for no

Compound	Cell line		
	LNCaP +DHT	LN-AR +DHT	LN-AR -DHT
1	500±100 nM	300±30 nM	120±30 nM
2	>10 μM	>10 μM	>10 μM
Bicalutamide	900 nM	>1 mM	(3.5 μM)

Table 4.1. IC_{50} values for inhibition of PSA mRNA expression. Values represent the average of IC_{50} determinations for three biological replicates, mean \pm s.d.

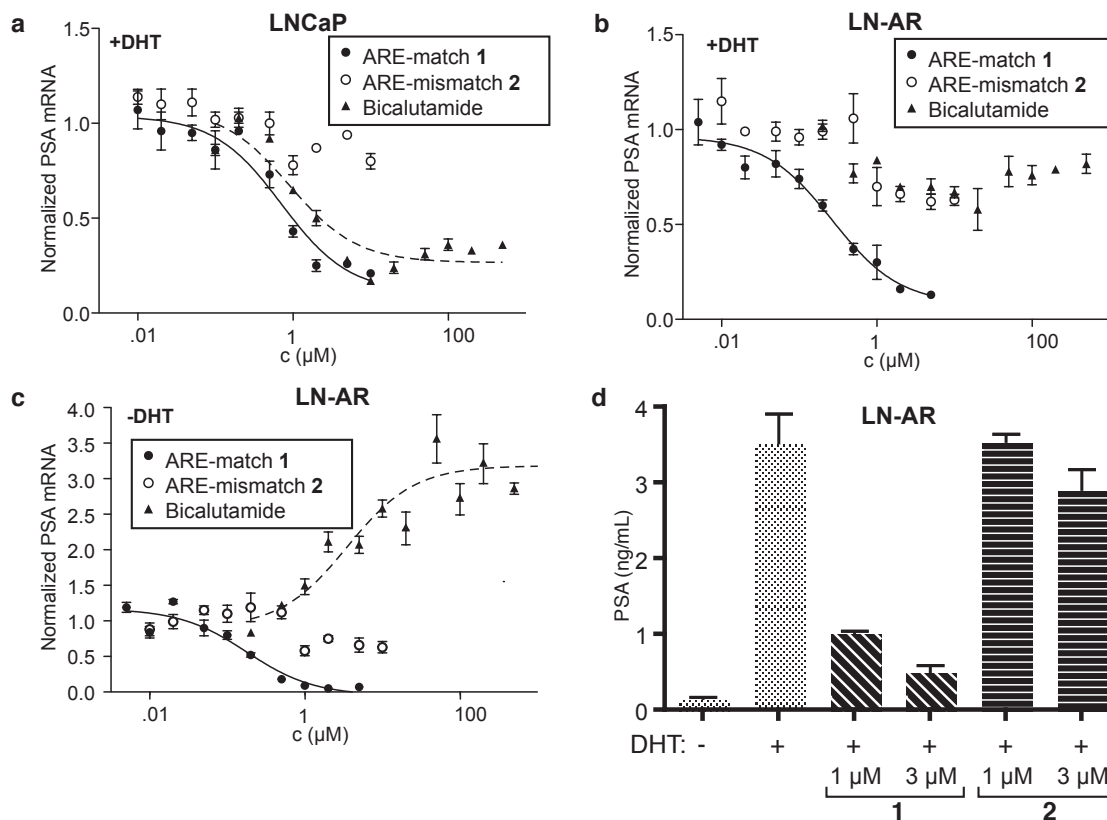


Figure 4.2. A Py-Im polyamide antagonist of AR-ARE binding inhibits expression of AR target gene PSA. (a) Dose-dependent inhibition of DHT-induced PSA mRNA expression by polyamide 1 and bicalutamide in hormone-sensitive LNCaP cells as measured by qRT-PCR analysis of cDNA (untreated, DHT-induced = 1). Inhibition by 2 is much less potent effective. (b) Inhibition of PSA mRNA expression in DHT-induced LN-AR cells (untreated, DHT-induced = 1). Bicalutamide and 2 are now similarly ineffective but compound 1 maintains its potency. (c) Effects of polyamides and bicalutamide on basal PSA mRNA expression in LN-AR cells (untreated = 1). Bicalutamide induces PSA expression under these conditions. Polyamide 2 is ineffective. (d) Effects of polyamides 1 and 2 on PSA protein expression in LN-AR cells as measured by ELISA. Compound 1 inhibits PSA production; compound 2 is less effective.

dose-dependent inhibition was observed with the mismatch compound. As expected, bicalutamide failed to inhibit DHT-induced PSA mRNA expression in LN-AR cells across a wide range of concentrations.

We also examined the polyamide's activity under non-induced conditions in charcoal-stripped media (no DHT). In this setting, bicalutamide induced PSA mRNA expression in accordance with the previously published result. The match polyamide again inhibited PSA expression, and the mismatch compound had little effect.

We confirmed the results of the mRNA expression analysis with an enzyme-linked immunosorbent assay (ELISA) for PSA in LN-AR cell culture supernatants (Figure 4.2d).

The protein expression levels of PSA were also decreased for the match polyamide, and relatively unaffected for cells treated with the mismatch compound or with bicalutamide.

Polyamide treatment does not reduce AR occupancy at AREs in LN-AR cells.

Previously, we have shown that compound **3** downregulates the expression of the AR-responsive gene FKBP5 in DHT-stimulated LNCaP cells at 10 μ M. Additionally, this compound decreased AR occupancy at the FKBP5 intronic ARE to 50% of DHT-induced levels. This locus was chosen as a test case for polyamide disruption of AR binding in the nuclei of live cells because of its particularly strong signal and large dynamic range between the non-induced and induced states. The original data obtained with 10 μ M compound **3** in DHT-stimulated LNCaP cells have been reproduced in LNCaP cells alongside the results obtained with 3 μ M compound **1** (Figure 4.3). However, in LN-AR cells, neither compound was able to block AR binding to this locus. LN-AR cells treated with 10 μ M of the third-generation, synthetic anti-androgen RD162 did behave as expected; AR bound to this compound is excluded from the nucleus and thus ARE

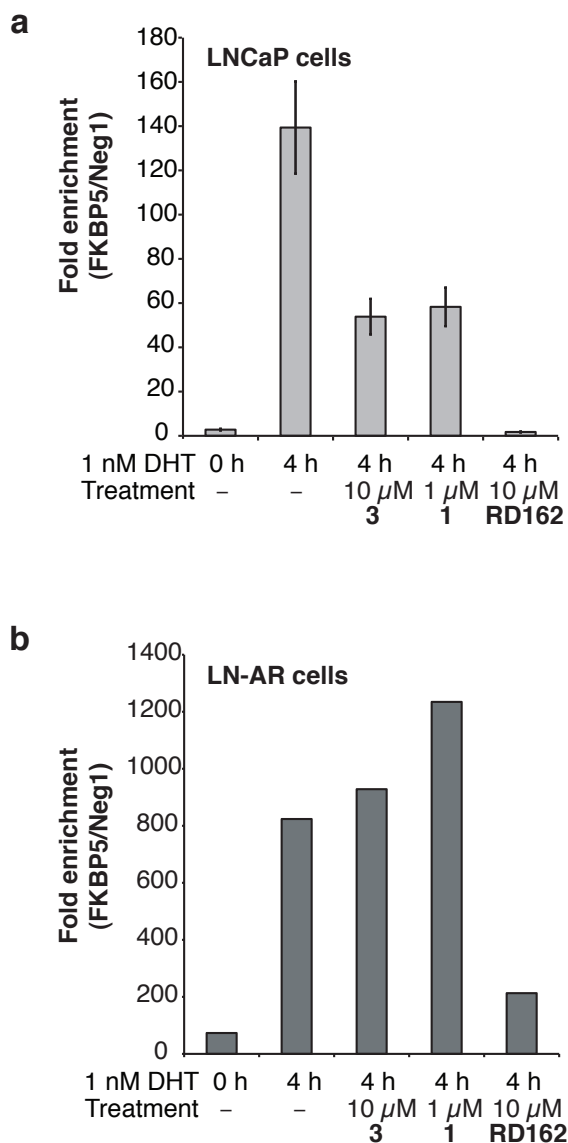


Figure 4.3. Inhibition of AR occupancy at the FKBP5 intronic enhancer. (a) Chromatin immunoprecipitation (ChIP) experiment in LNCaP cells showing inhibition of ARE occupancy by ARE-targeted polyamides **3** and **1**, as well as RD162. The data represent the mean and s.d. of three independent pulldowns. (b) AR ChIP in LN-AR fails to show any inhibition of AR occupancy by **3** or **1**. RD162 retains its activity. The data represent the mean of two independent pulldowns and are representative of 3 biological replicates.

occupancy is reduced to the non-induced state.

4.2.3. Inhibition of AR-target gene expression is accompanied by cytotoxicity.

In addition to targeted compounds that interrupt AR signaling, cytotoxic DNA-binding compounds can also disrupt the AR axis (10). To look for polyamide-mediated cytotoxicity, we first performed a cell growth assay in both LNCaP and LN-AR cells with compounds **1**, **2**, at 1 μM and bicalutamide at 10 μM concentration (Figure 4.4A). Growth inhibition caused by **1** became apparent after 72 hours continuous treatment, as measured by manual hemocytometry.

The cell growth assay indicated that compound **1** was cytostatic at 1 μM after 72 hours. To look for a cytotoxic effect and to define the cytotoxic concentration, we treated LNCaP and LN-AR cells with increasing concentrations of compound **1** for 72 and 96 hours (Figure 4.4B). The surviving cells were stained with sulfarhodamine B and quantitated by spectrophotometry. The compound showed dose-dependent cytotoxicity in

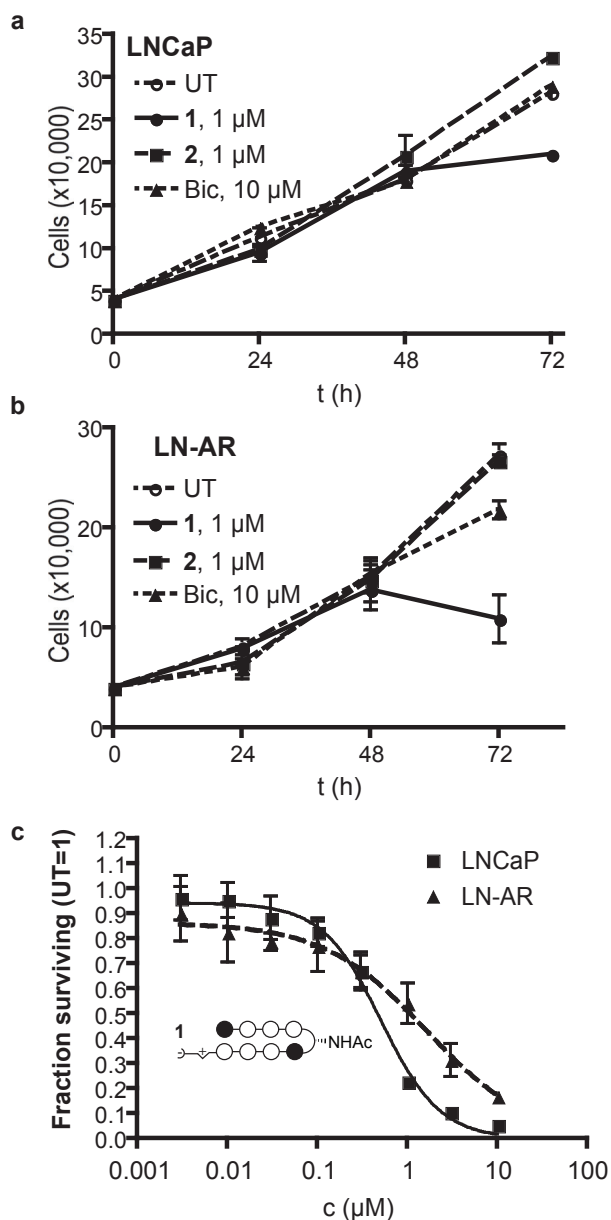


Figure 4.4. Inhibition of prostate cancer cell growth and cytotoxic response following treatment with ARE-targeted polyamide **1**. (a) Growth curve of LNCaP cells treated with either **1** (1 μM), **2** (1 μM), or bicalutamide (10 μM). Compound **1** displays a cytostatic effect at 72 h. (b) Growth curve of LN-AR cells treated with either **1** (1 μM), **2** (1 μM), or bicalutamide (10 μM). Compound **1** displays a slight cytotoxic effect at 72 h. (c) Cytotoxicity assay displaying dose-responsive cell death in response to **1** in LNCaP and LN-AR cells at 96 h. as measured by sulfarhodamine B staining.

both cell lines. The data were fit with non-linear least squares analysis to give the IC_{50} values shown in Table 4.2.

Cell line	AR	Cytotoxicity IC_{50} values (μM)	
		72h	96h
LNCaP	+	1.8 ± 0.9	0.6 ± 0.2
LNAR	+++	3 ± 1	1.5 ± 0.2

Table 4.2. Cytotoxicity IC_{50} values in LNCaP and LN-AR cells in response to treatment with **1**.

4.2.4. Polyamide treatment of LN-^a

AR cells activates cell death and cell stress response pathways on the same timescale as PSA down-regulation. The cell death response to cytotoxic compounds generally follows either a necrotic or apoptotic pathway. To look for induction of apoptosis (11), we treated LN-AR cells with $1 \mu M$ **1** or $1 \mu M$ **2** for up to 96 hours using a luciferogenic substrate for Caspases 3 and 7 (Figure 4.5A). Cells treated with **1** showed a time-dependent increase in Caspase 3/7 activity starting at 48 hours. Untreated cells and cells treated with **2** showed no such induction.

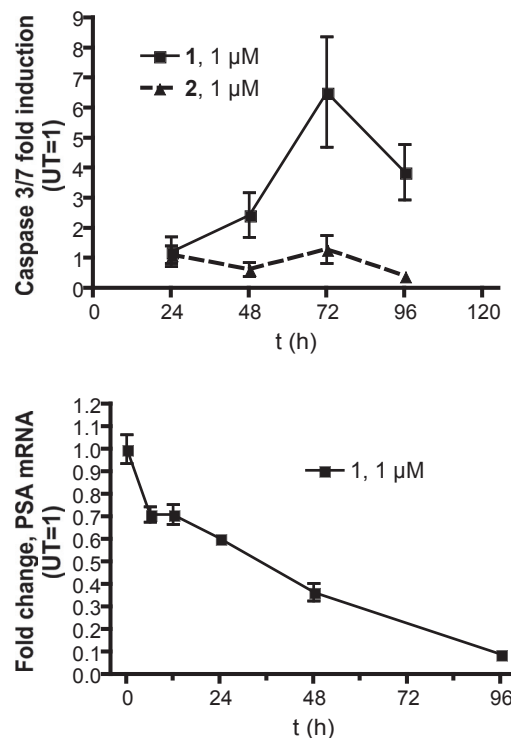


Figure 4.5. Caspase 3/7 activation accompanies PSA downregulation in unstimulated LN-AR cells. (a) Time course of Caspase 3/7 fold induction (UT=1) following treatment with $1 \mu M$ match polyamide **1** or $1 \mu M$ mismatch polyamide **2** as measured by cleavage of a luciferogenic substrate for Caspases 3 and 7. Activity is first detectable at 48 h. and peaks at 72 h. (b) Time course of fold changes (UT=1) in PSA mRNA levels as measured by RT-PCR. Inhibition of basal PSA expression is detectable after 6 h of treatment and continues to decline for the entire time course.

To examine the effects of polyamide inhibition of PSA mRNA expression on the same time course as the caspase induction, we treated unstimulated LN-AR cells in rich media with 1 μM **1** and measured mRNA levels at intervals up to 96 hours (Figure 4.5B). This assay measures inhibition of PSA from the steady state; cell culture media contains dilute concentrations of steroid hormones, so there is some basal expression of AR-target genes. In this assay, we observed time-dependent inhibition of PSA expression. Inhibition was detectable even after 6 hours of polyamide treatment and expression continued to decline until the 96-hour time point. Measurement of PSA inhibition in DHT-stimulated cells was performed after 64 hours incubation with the polyamide, a timepoint at which unstimulated, polyamide-treated cells showed significant Caspase 3/7 activity.

The tumor suppressor protein p53 can be activated by a wide array of stressors, including DNA damage and replicative or oxidative stress (12). In response to stress, p53 accumulates as it is rescued from ubiquitination and degradation. To look for accumulation of p53 in response to polyamide treatment, we treated LN-AR cells with 1 μM **1** for up to 96 hours and assayed total cellular lysates for accumulation p53 by sandwich ELISA (Figure 4.6). A standard curve using purified protein was created for this and the subsequent

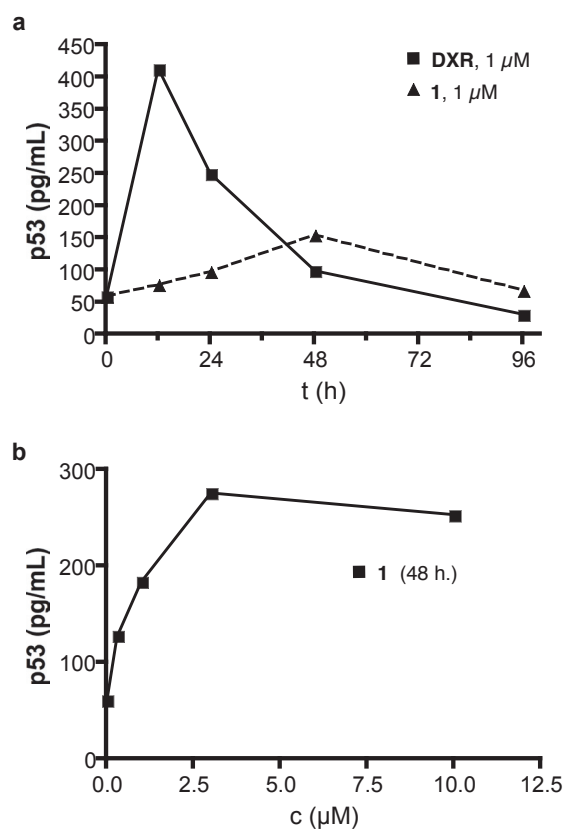


Figure 4.6. Stabilization of p53 in response to polyamide treatment. (a) Time course of p53 stabilization in LN-AR cells treated with 1 μM doxorubicin (DXR) or 1 μM **1** as measured by sandwich ELISA. Levels of p53 peak at 48 h. in cells treated with polyamide **1**. (b) Dose-response of p53 stabilization in LN-AR cells treated with increasing concentrations of **1** as measured by sandwich ELISA. Levels of p53 peaked at ~6-fold induction at 3 μM **1**.

experiment. Cells treated with 1 μ M doxorubicin, a DNA-damaging agent known to induce p53 stabilization, were included as a positive control. The polyamide-treated cells showed a time-dependent induction of p53 that peaked at 48 hours. The induction was weak when compared to that produced by doxorubicin. To look for a dose-dependent effect, LN-AR cells were treated with increasing concentrations of **1** for 48 hours and then subjected to the same ELISA. The induction of p53 was dose dependent and appeared to plateau at 3 μ M. Maximal induction was about 4-fold over the untreated condition ($t = 0$).

4.3. Discussion

In the hormone-resistant LN-AR cells, the ARE-targeted polyamide **1** maintains potency against PSA mRNA expression (Figure 4.1) but fails to decrease the binding of AR to the ARE in the FKBP5 intronic enhancer (Figure 4.2). The original polyamide antagonist, compound **1** also fails to inhibit AR binding to this locus in LN-AR cells. The LN-AR cell line is an LNCaP daughter cell line that contains a stably integrated construct that overexpresses AR. It is unclear how this single change would prevent polyamide-mediated inhibition of AR binding without disrupting the compound's ability to inhibit AR-driven gene expression.

It is possible that the polyamide and AR can co-occupy the FKBP5 intronic ARE when AR expression levels are raised to several times wild-type levels. Since AR binds in the major groove and the polyamide in the minor groove, AR inhibition by this compound is presumed to be allosteric. The transactivating function of AR is known to be allosterically sensitive, and there is precedent for a DNA-bound, transcriptionally inactive state of AR (13). Exploration of polyamide and receptor co-occupancy would be greatly facilitated by positive confirmation of polyamide binding to specific loci; the development of polyamide ChIP-seq methods is currently underway.

This study also revealed that treatment with **1** is accompanied by cytotoxicity, activation of the programmed cell death effector Caspases 3 and 7 (Figures 4.3 and 4.4). Further investigation of these findings should include an examination of their AR dependence, as hormone withdrawal is also known to induce an apoptotic cell death response in LNCaP cells (14). PC-3 and DU145 are prostate cancer cell lines that express very low levels of AR and display androgen-independent growth (15); these would make excellent model systems for further experimentation.

An additional consideration is that the observed effects on AR-driven gene expression could be a result of the cytotoxic insult. In particular, the data suggest a role for

p53 in the observed downregulation of AR-target gene expression. When p53 is stabilized in LNCaP cells, it acts as a repressor of AR homodimerization and suppresses expression of the AR gene(16,17). Additionally, it can bind a response element in the PSA promoter and repress its transcription (18). Experiments with polyamides **1** and **3** with p53 knockdown constructs in LNCaP cells could define the dependence of the observed transcriptional inhibition and cytotoxicity on p53 signaling.

4.4. Materials and methods

4.4.1. Compound synthesis. Polyamides **1**, **2**, and **3** were synthesized synthesized by solid-phase methods on Kaiser oxime resin (Nova Biochem, Darmstadt, Germany) according to established protocols (19). 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 described. Purities and identities of the polyamides were assessed by HPLC, UV-visible spectroscopy, and MALDI-TOF MS. RD162 was synthesized by microwave-assisted solution-phase synthesis according to previously published protocols (9). Purity and identity of RD162 were assessed with ¹HNMR and ESI-MS. Doxorubicin was purchased from Sigma-Aldrich and used without further purification, as were all other compounds and supplies unless otherwise specified.

4.4.2. Cell culture. LNCaP and LN-AR cells were maintained in RPMI 1640 with 10% FBS at 37°C under 5% CO₂. LNCaP cells were purchased from ATCC (Manassas, VA). LN-AR cells were a gift from C.L. Sawyers at Memorial Sloan-Kettering Cancer Center (NY, NY).

4.4.3. Measurement of PSA mRNA and Protein. 40,000 LNCaP or LN-AR cells were plated in RPMI1640 (Invitrogen) supplemented with 10% FBS (Irvine Scientific, Santa Ana, CA). For DHT-induced experiments, the medium was replaced with RPMI medium 1640 containing 10% charcoal-stripped FBS after 72 h with or without polyamides at the designated concentrations. Cells were grown for an additional 48 h and then treated with 1 nM DHT for 16 h. When appropriate, bicalutamide was added 2 h before DHT stimulation. For assays of basal PSA expression in normal media, 40,000-80,000 cells were plated in 24-well plates and compounds were added in fresh, complete media after 24 h. Isolation of

RNA and cDNA synthesis was performed as described (20). Quantitative real-time RT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI 7300 instrument. PSA mRNA was measured relative to β -glucuronidase as an endogenous control. Primer sequences are available upon request. Cell-culture supernatants were collected for an ELISA (R & D Systems, Minneapolis, MN) to measure PSA protein according to the manufacturer's protocol.

4.4.4. Chromatin immunoprecipitation. ChIP analysis of AR occupancy at the FKBP5 intronic ARE was performed as described using enrichment at a negative locus for normalization (21). Briefly, 80×10^6 LNCaP or LN-AR cells were plated in RPMI1640 containing 10% charcoal-treated FBS. Polyamides were added after 24 h and allowed to incubate for an additional 48 h. RD162 was added 2 h prior to induction with DHT. DHT was added and the cells were harvested after 4 h. Workup and analysis of fold enrichment were performed as described, using a negative locus for quantitation of immunoprecipitated DNA using qPCR.

4.4.5. Growth inhibition assay. 40,000 LNCaP or LN-AR cells were plated in triplicate in 24-well plates. Compounds and controls were added after 24 hours pre-incubation. At each time point, cells were trypsinized, pelleted by centrifugation and resuspended in 1 mL complete media. Cells were hand counted with a hemocytometer. The data represent the mean and standard deviation of these technical replicates.

4.4.6. Cytotoxicity assays. IC_{50} values for cytotoxicity were determined using a previously described, sulfarhodamine-based, colorimetric assay for cellular protein content in 96-well microplates (22). LNCaP or LNAR cells were plated at 3,000 or 4,000 cells per well for the 72 h and 96 h timepoints, respectively. Compounds were added in 100 μ L RPMI1640 supplemented with 10% FBS 24 h after plating. Quadruplicate wells were used for each

concentration. At the appropriate time, the cells were fixed with 100 μ L 10% trichloroacetic acid solution, washed, stained, and dried as described elsewhere.(22) After solubilization of the bound dye in 10 mM Tris, the absorbance was measured at 490 nm.

The data are charted as a percentage of untreated controls, corrected for background absorbance. IC_{50} is defined as the concentration that inhibits 50% of control cell growth. These values were determined by non-linear least squares regression fit to $Y = A + (B-A)/(1+10^{((\text{Log EC}_{50}-X)*H})}$, where A=max., B=min., and H=Hill Slope. Three independent trials were averaged; stated IC_{50} values represent the mean and standard deviation. These calculations were performed using Prism 4 (GraphPad) software.

4.4.7. Apoptosis assays. LN-AR cells were plated in 96-well microplates at 4,000 cells per well. As above, compounds and controls were added 24h after plating. Each time point was assayed in triplicate. At harvest, Caspase 3/7 activity was assessed using 100 μ L of Caspase-Glo reagent (Promega), which contains the proluminescent caspase substrate DEVD-aminoluciferin. Luminescence was measured after 30 minutes incubation at room temperature. Luminescence data are expressed as the mean and standard deviation of technical replicates in arbitrary units.

4.4.8. P53 stabilization assay. 400,000–800,000 LN-AR cells were plated in 10 cm diameter dishes. Compounds were added after 24h and were allowed to incubate for various times up to 72 hours. At harvest, cells were washed once with PBS then treated with 1 mL ice-cold lysis buffer and collected with a rubber policeman. After 15 min incubation at 5°C, lysates were centrifuged at 4,000 x g, reserving the supernatants. Protein concentrations were determined by Bradford assay (Promega). P53 protein was assayed by sandwich ELISA (R&D Biosystems) and performed according to the manufacturer's recommendations. 10 μ g total protein was used for each experimental sample. A standard curve using the provided purified p53 protein was created for each assay run, and p53 levels

for each experimental sample were determined by interpolation. The data are expressed as the mean of two technical replicates.

4.5. References.

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