Chapter 3

ACTIVITY OF A PY-IM POLYAMIDE TARGETED
TO THE ESTROGEN RESPONSE ELEMENT

The text of this chapter was taken in part from a manuscript co-authored with Nicholas G. Nickols, Amanda E. Hargrove, Benjamin C. Li, Jevgenij A. Raskatov, and Peter B. Dervan.

(Nickols NG*****, **Szablowski JO***, Hargrove AE, Li BC, Raskatov JA, Dervan PB. "Activity of a Py-Im Polyamide Targeted to the Estrogen response Element," *Mol. Cancer Ther.*, **12**:675-684, (2013). *denotes equal contribution)

Abstract

Pyrrole-imidazole (Py–Im) polyamides are a class of programmable DNA minor groove binders capable of modulating the activity of DNA-binding proteins and affecting changes in gene expression. Estrogen receptor alpha (ERα) is a ligand-activated hormone receptor that binds as a homodimer to estrogen response elements (ERE) and is a driving oncogene in a majority of breast cancers. We tested a selection of structurally similar Py–Im polyamides with differing DNA sequence specificity for activity against 17β-estadiol (E2)–induced transcription and cytotoxicity in ERα positive, E2-stimulated T47DKBluc cells, which express luciferase under ERα control. The most active polyamide targeted the sequence 5′-WGGWCW-3′ (W = A or T), which is the canonical ERE half site. Whole transcriptome analysis using RNA-Seq revealed that treatment of E2-stimulated breast cancer cells with this polyamide reduced the effects of E2 on the majority of those most strongly affected by E2, but had much less effect on the majority of E2-induced transcripts. *In vivo*, this polyamide circulated at detectable levels following subcutaneous injection and reduced levels of ER-driven luciferase expression in xenografted tumors in mice after subcutaneous compound administration without significant host toxicity.

Introduction

Estrogen receptor-alpha (ERα) is a member of the nuclear hormone receptor family of transcription factors and is active in a majority of breast adenocarcinomas (1, 2). Breast tumors that express ERα and are sensitive to circulating estrogens respond to therapeutics that modulate ERα activity (3). Such therapeutics include tamoxifen, a selective ER modulator that acts as a weak agonist/antagonist by binding to the ERα ligand-binding pocket and the aromatase inhibitors that inhibit synthesis of E2 (3). A different strategy for modulation of ERα activity is inhibition of the ERα–ERE interface by a DNA-binding molecule.

Pyrrole-imidazole (Py–Im) polyamides are a class of synthetic, minor groove-binding ligands inspired by the natural product distamycin A (4, 5). Py–Im polyamides are oligomers of aromatic amino acids linked in series to fold in an antiparallel fashion when bound in the minor groove of DNA (4, 5). Sequence specificity is programmed through side-by-side pairings of the Py and Im subunits that recognize differences in the shape and hydrogen bonding pattern presented by the edges of the Watson–Crick base pairs in the floor of the minor groove (6, 7). Binding specificity has been extensively characterized by DNAse I footprinting titrations and other methods. An Im:Py pair preferentially recognizes G:C; Py:Im prefers C:G, and Py:Py is degenerate for A:T and T:A (6, 7). Py–Im polyamide binding in the minor groove also induces allosteric changes to the major groove (8, 9), and binding affinity is sufficient to modulate the binding of DNA-binding proteins (8–11).

In cell culture, selected polyamides have been used to modulate expression of genes induced by testosterone (11), TNF-α (12), hypoxia (13, 14), and dexamethasone (10). The mechanisms by which polyamides affect gene expression changes in cell culture are still

not well understood and may involve direct effects on multiple DNA-dependent processes including transcription factor occupancy, chromatin structure, RNA polymerases, and DNA replication (15). The pharmacokinetics and toxicity of a number of polyamides after intravenous and subcutaneous injection in mice and rats have been described (16–18). In mice, a selected polyamide was reported to induce changes in TGF-β expression in kidney glomeruli, and a fluorescent analog of this polyamide was observed in kidney glomeruli after tail vein injection in rats (19). Gene expression changes have also been observed in tumor xenografts in immune-compromised mice treated with a hairpin-polyamide (20).

In this study, our goal was to identify a Py–Im polyamide capable of affecting E2-stimulated gene expression in breast cancer cells and characterize its activity in cell culture and in tumor xenografts. To do this, we drew from an earlier study that reported a polyamide targeted to the estrogen response element (ERE) consensus half site 5′-WGGWCW-3′ inhibited ERα–binding to DNA in cell-free systems (21). The DNA-binding affinity and specificity of the ERE-targeted polyamide was characterized in this and other studies (21, 22). Since those publications, we have improved the nuclear uptake of polyamides via modification of the C-terminus (23). We have also shown that polyamides are bioavailable after intravenous and subcutaneous injection in mice (20, 24). We then decided to reexamine the activity of polyamides capable of disrupting ERE-driven gene expression for use *in vivo*. We have screened a focused library of polyamides for cytotoxicity and inhibition of luciferase activity using the breast cancer cell line T47D-KBluc that expresses luciferase under the control of 3 tandem, canonical EREs (25). The most active polyamide identified, which targets the consensus ERE, was further evaluated

and showed a partial suppression of E2-stimulated gene expression in cell culture. This polyamide circulated in mouse serum after subcutaneous injection and showed activity against E2-induced luciferase expression in T47D-KBluc tumor xenografts in mice with minimal host toxicity. A fluorescent analog of this polyamide distributed widely in both tumor and mouse tissue after subcutaneous injection.

Materials and Methods

Polyamide synthesis and characterization

The polyamides **1** to **5** were synthesized following previously published solid phase synthesis protocols (26). Compound purities were confirmed by analytic high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry. Melting temperature analysis was conducted on a Varian Cary 100 spectrophotometer with temperature control. Oligonucleotides (IDTDNA) were dissolved in 10 mmol/L sodium cacodylate, 10 mmol/L KCl, 10 mmol/L $MgCl_2$, and 5 mmol/L $CaCl_2$ at pH 7.0 at a concentration of 2 μmol/L. Polyamides were added to oligo solution to a final concentration of 4 μmol/L in 0.1% dimethyl sulfoxide (DMSO). Oligonucleotides were annealed from 25°C to 90°C and then back to 25°C at 5°C/min. Subsequently, the temperature was elevated at a rate 0.5°C/min between 25°C and 90°C. Melting temperatures are defined as a maximum of the first derivative of absorbance at 260 nm over the range of temperatures.

Cell culture and imaging

Cell lines used were purchased directly from American Type Culture Collection (ATCC) and used within 6 months. No subsequent authentications were done by the authors. All

experiments were carried out with T47D-KBluc cells (ATCC), unless specifically mentioned otherwise. Cells were grown in RPMI-1640 and held at 37°C in 5% CO_2. Media was supplemented with 10% FBS and 1% penicillin/streptamycin. Before imaging, cells were plated in 35-mm optical dishes (MatTek) at 5×10^4 cells per dish in the presence of 10 nmol/L E2. Cells were dosed with polyamide for 24 hours. Cells were then washed twice with PBS and imaged on a confocal microscope (Exciter, Zeiss) using a ×63 oil immersion lens in a method previously described. Confocal imaging was conducted following our previously published protocols (27, 28).

Tissue processing for fluorescence imaging

The tissue sections for fluorescent imaging were obtained by fixing the tumors in 10% formaldehyde solution for 24 hours and subsequent cryoprotection in 15% sucrose (24 hours) and 30% sucrose (24 hours). The tumors were frozen in Tissue-Tek O.C.T. (Sakura Finetek) and 50 μm (for T47D-KBluc xenograft) or 10 μm (for other tissues) sections were obtained using a Leica CM 1800 cryotome. Imaging was conducted as described earlier.

Cell toxicity and luciferase assays

T47D-KBluc cells were plated at 3×10^3 cells per well in 96-well plates, incubated in standard growth media containing 10 nmol/L E2 for 48 hours, and then dosed with medium containing 10 nmol/L E2 and between 2 nmol/L and 50 μmol/L polyamides. The cells were then incubated for 96 hours and analyzed using either WST-1 assay (Roche) or luciferase assay system (Promega) according to the manufacturers' instructions.

Gene expression analysis by qRT-PCR

Cells were plated in 12 well-plates at 1.1×10^5 cells/well and incubated in the growth medium supplemented with 10 nmol/L E2 for 24 hours. Afterward, medium was exchanged with the growth medium supplemented with polyamides and 10 nmol/L E2. Quantitative real-time PCR (qRT-PCR) was conducted according to previously established protocols (3–6). Confirmation of inhibition of *TFF1* expression by polyamides 1 to 4 was carried out and qRT-PCR was conducted following the same timeline as cell toxicity and luciferase assays. Gene expression was normalized against *GUSB* as a housekeeping gene. All primers yielded single amplicons as determined by both melting denaturation analysis and agarose gel electrophoresis. The following primer pairs were used. *GUSB*: forward 5′-CTC ATT TGG AAT TTT GCC GAT T-3′; reverse 5′-CCC AGT GAA GAT CCC CTT TTT A-3′. *DOK7*: forward 5′-GAC AAG TCG GAG CGT ATC AAG-3′; reverse 5′-ATG TCC TCT AGC GTC AGG CT-3′. *WT1*: forward 5′-CAC AGC ACA GGG TAC GAG AG-3′; reverse 5′-CAA GAG TCG GGG CTA CTC CA-3. *TGFB2*: forward 5′-CAG CAC ACT CGA TAT GGA CCA-3′; reverse 5′-CCT CGG GCT CAG GAT AGT CT-3′.

Chromatin immunoprecipitation experiments

T47D-KBluc cells were plated into 500-cm^2 plates and grown in RPMI-1640 with 10% FBS until 75% confluence was reached. Plates were washed with RPMI-1640 with charcoal-treated 10% FBS, and then the media was replaced with RPMI-1640 with charcoal-treated 10% FBS with 2 μmol/L polyamide 1 and incubated for 48 hours. Plates were then treated with 10 nmol/L E2 or vehicle for 45 minutes. Cross-linked chromatin was obtained using the 2-step cross-linking methods previously described (29). Chromatin

was isolated and sheared. Antibodies to ERα (AC-066-100; Diagenode) were used to immunoprecipitate ERα–bound DNA fragments. Cross-links were reversed and PCRs using primers targeted to the regions of interest were used to assess enrichment of bound fragments as compared with negative controls. *TFF1* promoter: forward 5′-TCA GAT CCC TCA GCC AAG AT-3′; reverse 5′-TGG TCA AGC TAC ATG GAA GG-3′. Negative loci control: forward 5′-AAA GAC AAC AGT CCT GGA AAC A-3′; reverse 5′-AAA AAT TGC TCA TTG GAG ACC-3′.

Circulation and toxicity in vivo

All animal experiments were carried out according to approved Institutional Animal Care and Use Committee protocols at the California Institute of Technology (Pasadena, CA). Circulation studies were done as previously described (30). Briefly, 120 nmol of polyamide 1 was injected subcutaneously into the right flank of 4 female C57BL/6 mice in a total of 200 μL of a 20% DMSO/PBS vehicle. Blood was collected retroorbitally at serial time points. Serum was treated with methanol, analyzed via HPLC, and quantified against a standard curve of concentration versus peak area, all as previously described, to determine approximate serum concentrations (24). For toxicity studies, 5 female C57BL/6 mice were injected with 20 nmol of polyamide 1 in a total of 200 μL of a 20% DMSO/PBS vehicle on days 1, 3, 5, 8, 10, 12, and then with 30 nmol on days 15, 17, 19, 22, 24, and 26 and were weighed before each treatment day. Mice were euthanized if weight loss was more than 15% of initial body weight, if dehydration was more than 10%, or if moribund behavior was observed. None were observed in this experiment.

Engraftment of T47D-KBluc.

Experiments were carried out in appropriately shaved female NSG mice (JAX) between 8 and 12 weeks of age. Cells were injected into the left flank area of the animals as suspensions of 5.0×10^6 mL^{-1} in 50% RPMI-1640 growth medium and 50% Matrigel, 200 µL per injection. Mice also received a subcutaneous E2 pellet (0.72 mg, 60-day slow release; Innovative Research of America) implanted into the right flank on the day of engraftment.

Treatment and tumor monitoring.

Mice were treated with either 25 nmol of polyamide 1 or 50 nmol of polyamide **5**. For the short-term and fluorescent imaging studies, they were treated for 8 days after engraftment and every second day for a total of 4 injections. For long-term treatment, injections started 16 days after engraftment and were continued twice a week for the following 4 weeks. Imaging was accomplished using the IVIS Imaging System (Caliper). The animals were anesthetized with 2% to 3% isoflurane and injected intraperitoneally with 150 µL of RediJect D-luciferin (Caliper) and subsequently transferred to the imaging chamber, where isoflurane levels were reduced to 1% to 2.5%. The floor of the imager was heated to +37°C to avoid animal hypothermia. Breathing frequency was monitored and not allowed to drop below 1 per second, adjusting the isoflurane levels accordingly at all times.

Endpoint criteria and euthanasia.

Animal endpoint criteria encompassed weight loss of more than 15%, restriction of motoric function by the engrafted tumor, dehydration of more than 10%, and moribund behavior. Where appropriate, the animals were euthanized by asphyxiation in a CO_2 chamber.

Tumor tissue harvest.

Animals were resected and tumors excised using standard forceps, scissors, and surgical blades. The tumors were weighed immediately afterward. For studies with fluorescein isothiocyanate (FITC)-conjugate 5, resected tumor tissue was homogenized via blunt force and then pushed through a microfilter to achieve single cell suspensions, which were plated on glass microscopy slides for 6 hours before imaging using a Zeiss Exciter fluorescence confocal microscope.

RNA-Seq sample preparation and data processing

Cells for gene expression analysis were plated in 10-cm diameter dishes at 1.1×10^6 cells per dish and incubated in the growth medium supplemented with 10 nmol/L E2 for 24 hours. Afterward, medium was exchanged with the growth medium supplemented with polyamides and 10 nmol/L E2 and incubated for 48 hours in 5% CO_2 and 37°C. The RNA was then harvested using an RNEasy Kit (Qiagen). Subsequently, a Riboguard RNAse inhibitor was added and samples were treated with TurboDNA Free DNAse (Ambion), according to manufacturers' instructions. RNA-Seq libraries were prepared using standard Illumina reagents and protocols. Single read sequencing with the read length of 50 nucleotides were conducted on the Illumina HiSeq2000 sequencer, following manufacturers' instructions, producing 35 to 50 million reads per library. Sequencing data were mapped against the combined human (hg19) transcriptome, using the Bowtie program package 0.12.7 (31) and the refseq annotation. The open access processing package Cuffdiff was used to calculated differential gene expression. Inter-replicate statistical significance was calculated with the DEseq module (32).

Results

Design of polyamides

We synthesized 4 8-ring hairpin Py–Im polyamides to screen for activity against E2-stimulated gene expression (Fig. 3.1 and Fig. 3.2). Polyamide 1 targets 5′-WGGWCW-3′, which is the half site ERE consensus. Polyamide **2** was previously reported to inhibit a subset of dihydrotestosterone-induced gene expression in cultured prostate cancer cells (11). Polyamide 3 was recently characterized in cultured lung cancer cells and used to partially abrogate TNF-stimulated transcription (12). Polyamide 4 targets the sequence 5′ WGWCGW-3′. Polyamides 5 and 6 are FITC-conjugated analogs of polyamides 1 and 2, respectively, used to visualize cellular uptake and distribution in this study.

Figure 3.1 Ball-and-stick models of polyamides 1 to 6 with DNA target sequences as follows: 1, 5'-WGGWCW-3'; 2, 5'-WGWWCW-3'; 3, 5'-WGGWWW-3'; 4, 5'-WGWCGW-3'. Polyamides 5 and 6 are FITC-analogs of polyamides 1 and 2, respectively, used for fluorescence microscopy experiments. Chemical structures of polyamides 1 to 6 are in Fig. 3.2. IPA, isophthalic acid.

Evaluation of binding of polyamides to an ERE half site by DNA thermal denaturation assays

Polyamides 1 to 4 were incubated with duplex DNA 5′-CGATGGTCAAGC-3′, which contains an ERE half site consensus and melting temperatures measured (Fig. 3.3A). Duplex stabilization was greatest for polyamide 1, a polyamide that was predicted to bind this sequence based on established Py–Im polyamide pairing rules (6, 7). The other polyamides showed less stabilization of this duplex.
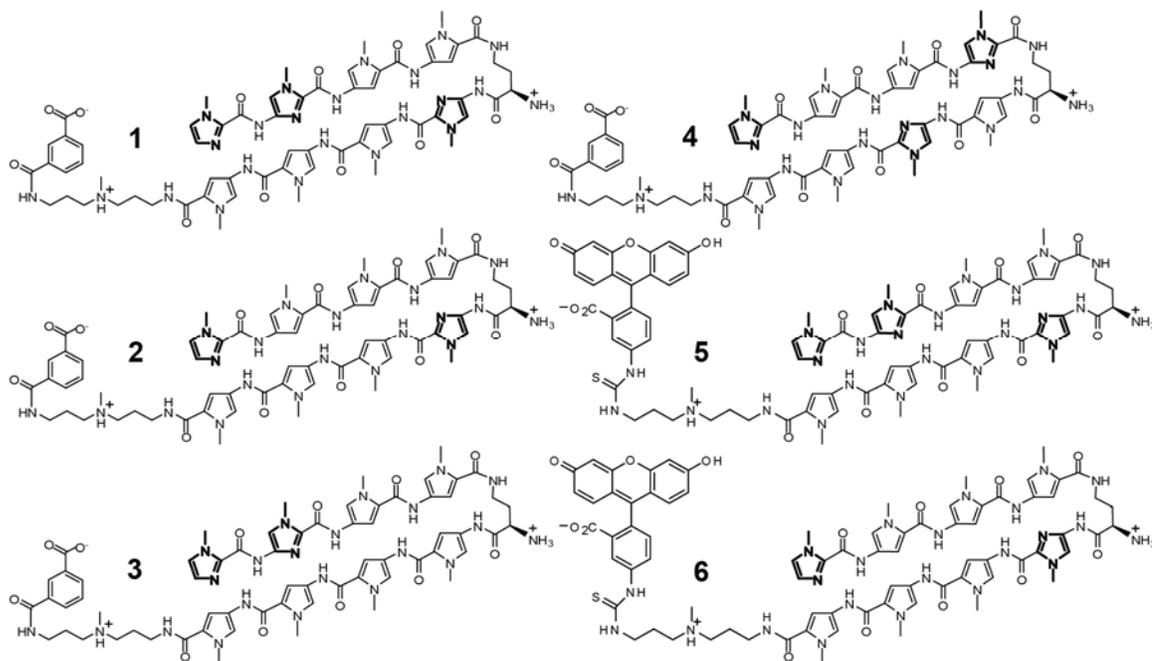
Figure 3.2 Chemical structures of compounds 1-6.

Luciferase activity and cytotoxicity in T47D-KBluc cells

The ERα–positive cell line T47D-KBluc expresses luciferase under the control of 3 tandem repeats of the sequence 5′-AGGTCACTTGACCT-3′ (25), which is the consensus sequence for the ERα–DNA homodimer (Fig. 3.3B). T47D-KBluc cells were grown in 10% FBS/RPMI-1640 media with 10 nmol/L E2 for 48 hours. Then, media was replenished with varying concentrations of polyamides 1 to 4 for 96 hours. An extended incubation time with E2 was used to approximate the *in vivo* condition of continued E2 circulation. Cell proliferation and viability was assayed using WST-1 (Roche) and luciferase output was measured (Fig. 3.3C). Both luciferase output and proliferation were affected most by treatment with polyamide 1 (IC_{50} 0.47 μmol/L for viability, 0.14 μmol/L for luciferase suppression) and least by polyamide 3 (IC_{50} > 2.5 and 1.5 μmol/L, respectively). The representative data for luciferase and WST-1 assay are shown in Fig. 3.4. We identified *TFF1* as one of the most highly induced transcripts by E2 based on published reports (33). The effects of polyamides 1 to 4 on E2-stimulated *TFF1* expression were measured to validate the luciferase screen. Polyamide 1 was again found most potent although polyamides 2 and 4 showed significant inhibition of *TFF1* as well (Fig. 3.3D). In addition, polyamide 1 shows significantly less toxicity to LNCaP, U251, and A549 cell lines (Fig. 3.5), which have low expression of ERα (34–37). Inhibition of *TFF1* mRNA by polyamide 1 is dose responsive (Fig. 3.6A) and chromatin immunoprecipitation of ERα at the *TFF1* promoter after E2 stimulation of cells pretreated with **1** showed reduced occupancy as compared with vehicle-treated cells (Fig. 3.6B, C).

A

| Polyamides | 5′ - CGA **TGGTCA** AGC -3′ | |
	T_m / °C	ΔT_m / °C
—	54.0 (±0.2)	—
1	69.1 (±0.3)	15.1
2	64.8 (±0.3)	10.8
3	63.5 (±0.5)	9.5
4	64.3 (±0.2)	10.3

B

ERE₂ — ERE₂ — ERE₂ ⟶ *LUCIFERASE*

ERE   ERE
5'-...**AGGTCA**CTGT**GACCT**...-3'
3'-...**TCCAGT**GAC**ACTGGA**...-5'

ERE₂

C

Polyamides	ER-Luciferase IC_{50} (μmol/L)	Cytotoxicity WST-1, IC_{50} (μmol/L)
1	0.14 (± 0.04)	0.47 (± 0.17)
2	0.51 (± 0.15)	1.55 (± 0.63)
3	>1.5	>2.5
4	0.25 (± 0.05)	1.1 (± 0.2)

D
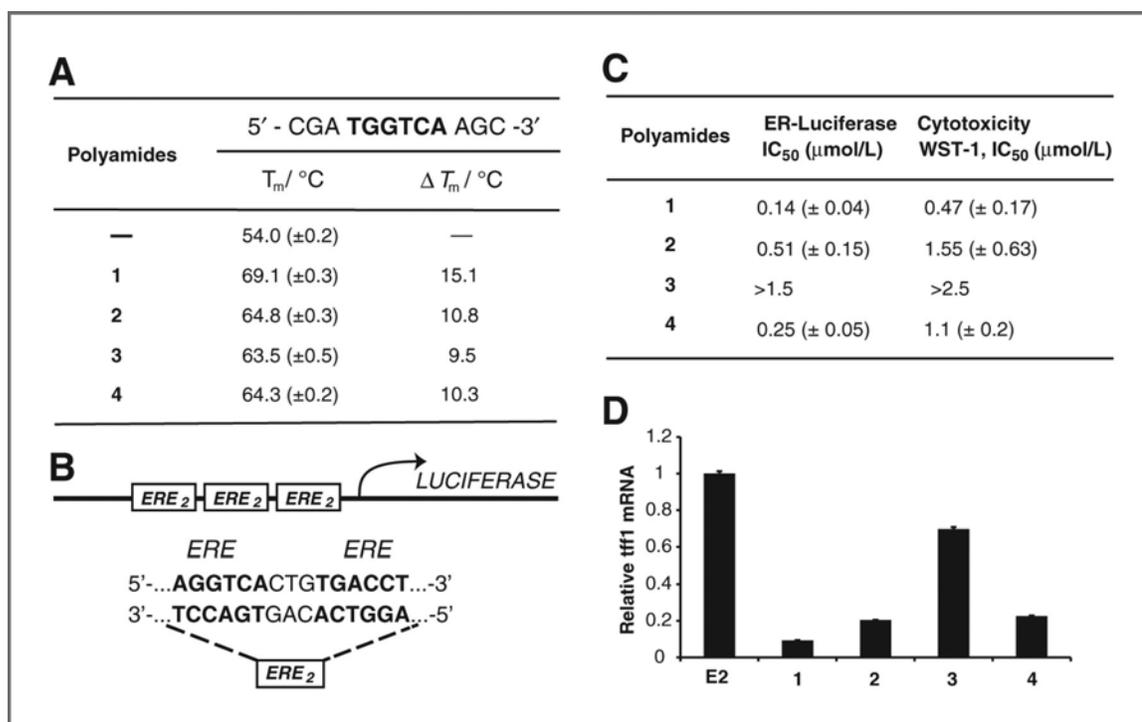
Figure 3.3 A, thermal denaturation assays of a duplex DNA oligonucleotide containing a half site ERE. Polyamide **1** shows the most stabilization. B, sequence of ERE-driven luciferase in T47D-KBLUC cells. C, polyamides **1** to **4** were screened for cytotoxicity and suppression of ER-driven luciferase. Polyamide 1 is most potent by both measures. Representative isotherms are displayed in Fig. 3.4. D, polyamides dosed at 0.3 μmol/L were screened for activity against TFF1 expression, a known ERE-driven gene. The relative activities of polyamides **1** to **4** approximately mirror what is seen in the luciferase assay at this concentration. At higher concentrations (∼1 μmol/L), all four polyamides show activity.
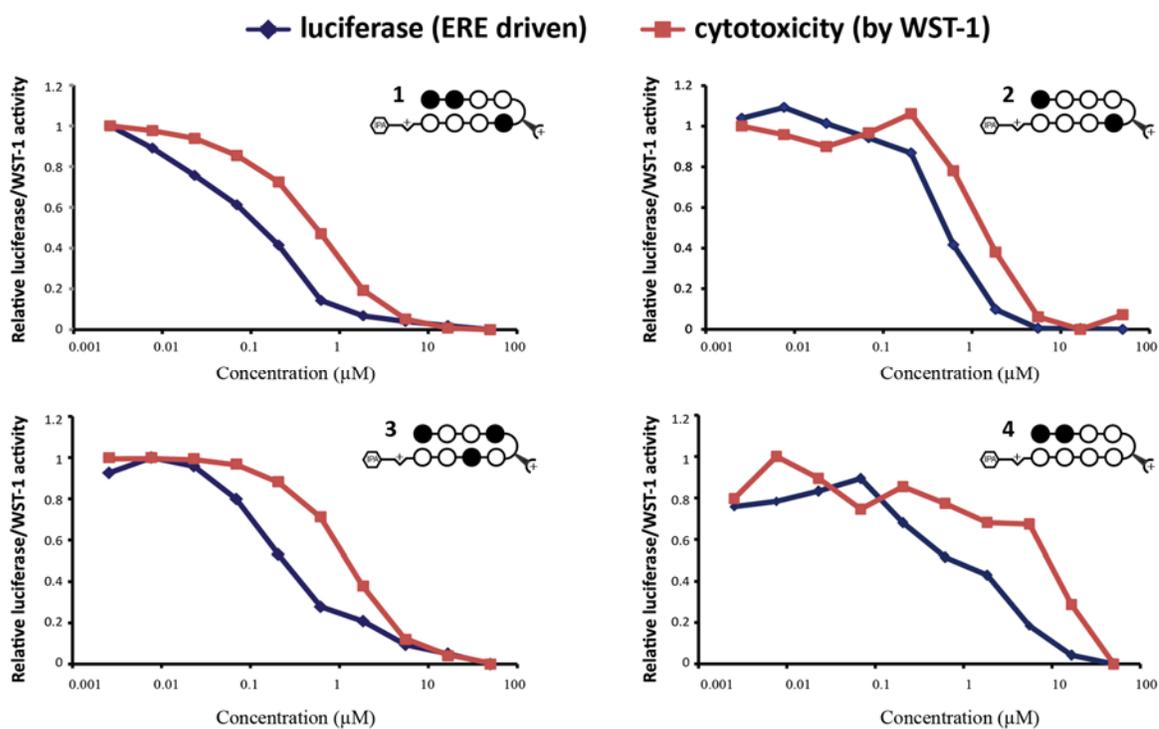
Figure 3.4. Representative data from luciferase and cytotoxicity (wst-1) assays for compounds 1-4.

Cell Line	IC50 (96h, **1**, m**M**)
T47D-KBLuc	0.47 ± 0.17
A549	8.5 ± 3.7
LNCaP	>50
U251	>50
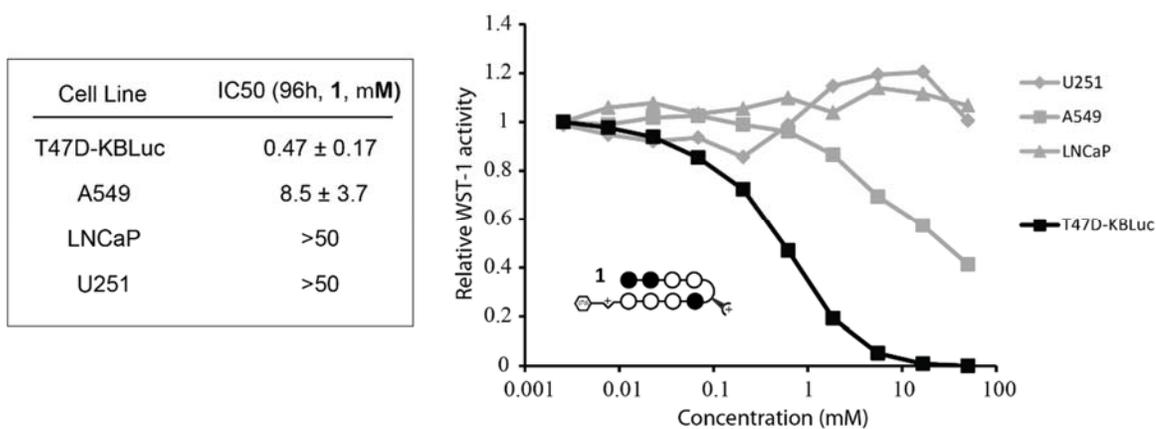
Figure 3.5 WST-1 cytotoxicity of 1 in T47D-KBLUC, LNCaP, A549, and U251 cells.
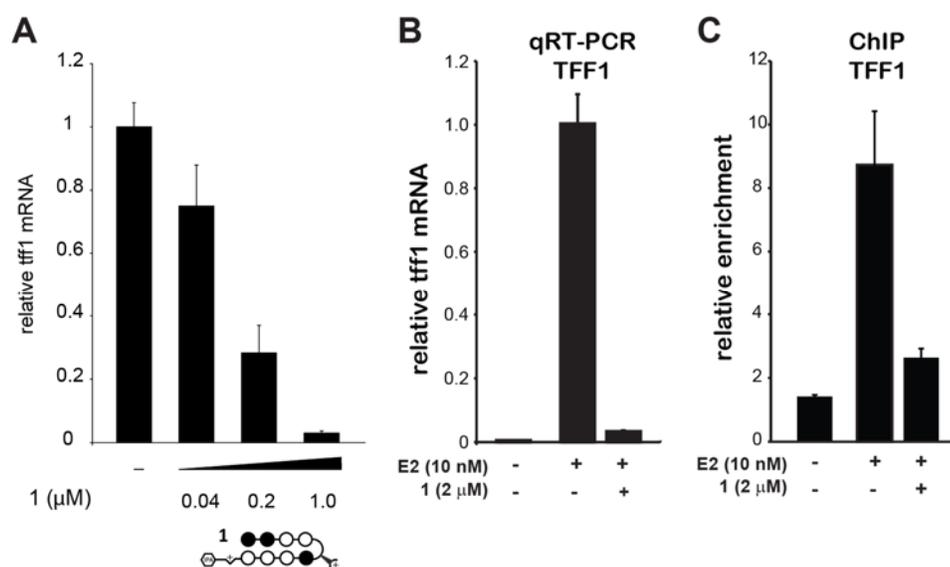
Figure 3.6 Quantitative RT-PCR analysis of Tff1 mRNA reduction after treatment with 1 for 96h is dose responsive. B, Relative mRNA of E2 induced tff1in the presence of 1 at 2 μM C, ERα occupancy at the tff1 promoter is reduced by 1. The cells were incubated with 1 for 48 hours prior to induction with E2 (10 nM) for 45 minutes. The IC50 for cytotoxicity of 1 at 48h in 10% CT-FBS/RPMI is 3.4 μM.

Genome-wide polyamide effects on E2-induced gene expression

Effects of hairpin polyamide 1 at 0.3 and 1 μmol/L on the transcriptome of E2-induced cells were measured using RNA-Seq. Reads were mapped using Hg19 reference human genome and data were analyzed using the Bowtie and CuffDiff packages (38). Only the genes with fragments per kilobase of exon per million fragments mapped (FPKM) ≥ 20 and at least 2-fold change in gene expression upon treatment with either polyamide 1 or E2 were used in the analysis (Table 3.1). Among those genes, at 1.0 μmol/L, polyamide 1 affected expression of 346 genes (0.7% of total) at least 2-fold as compared with E2-treated control. Of these genes, an equal number of genes were up- and downregulated (173 in each case). At the lower concentration of 0.3 μmol/L, expression of 127 genes (0.3% of total) was affected at least 2-fold, and a majority of these genes (77 vs. 50) were

downregulated. At the same threshold, E2 upregulated 1,003 genes (2.0%; Fig. 3.7A) and downregulated 575 genes (1.2%; Fig. 3.7B). A fraction of expression changes induced by E2 were reversed by polyamide 1 (Table 3.2), and this fraction was greater for E2-repressed genes. Among E2-upregulated genes, 43 (4.3%) were repressed by polyamide 1 at least 2-fold at 1.0 μmol/L. Among those 575 genes that were downregulated by E2, 95 (16.5%) were derepressed by **1** at 1.0 μmol/L at least 2-fold (Fig. 3.7A and B). Overall, of the 346 genes affected by polyamide 1 at 1.0 μmol/L, 138 (39.9%) represent genes whose up- or downregulation by E2 was abrogated by polyamide treatment. Genes whose expression was affected by polyamide 1 at a lower concentration (0.3 μmol/L) were largely a subset of the genes affected at 1.0 μmol/L, 103 of which (81.1%) were affected by polyamide 1 at both concentrations.

Further analysis was conducted using Euclidian distance clustering with complete linkage (Fig. 3.7C). Interestingly, while the majority of E2-affected genes are not affected by polyamide 1, out of the top 50 genes most strongly affected by E2, 28 (56%) are inhibited at least 2-fold and 38 of 50 (78%) genes are inhibited at least 1.5-fold by polyamide 1 (Fig. 3.7D). Five transcripts were selected for verification by qRT-PCR and all five showed good reproducibility of the expression changes seen by RNA-Seq (Fig. 3.8). Four were upregulated by E2 (*AREG*, *DOK7*, *TFF1*, and *WT1*) and one downregulated by E2 (*TGFB2*).

Circulation and toxicity of polyamide 1 in mice

To assess serum concentrations of **1** after subcutaneous injection, 4 female C57BL/6 mice were injected subcutaneously into the left flank with 120 nmol of polyamide 1 in a

200 µL 20% DMSO/PBS vehicle. Serial serum samples were taken via retroorbital draw

and processed by methods previously described (30). Polyamide 1 was detectable in serum

for up to 24 hours after injections, and reached a maximum concentration of 3 µmol/L at 6

hours after injection (Fig. 3.9A). Toxicity after repeated injections of **1** was assessed by

daily weights and visual inspection of treated mice. Five female C57BL/6 mice were

injected with 20 nmol of polyamide 1 subcutaneously to the left flank 3 times a week for 2

weeks without measurable weight loss. The regimen was then increased to 30 nmol for 2

weeks, again without measurable weight loss or changes in animal behavior (Fig. 3.9B).
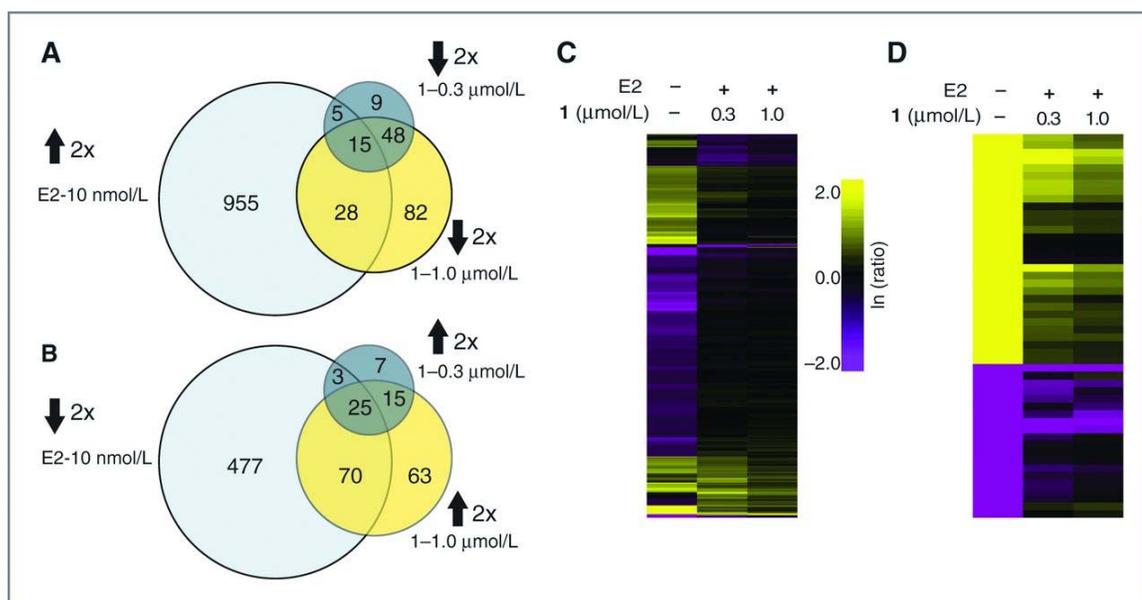
Figure 3.7 RNA-seq global transcriptome analysis. All ratios are normalized to the induced control (10 nmol/L E2). A, Venn diagrams show the overlap between genes upregulated by E2 at least 2-fold and genes downregulated by polyamide 1 at 0.3 or 1.0 µmol/L. B, Venn diagrams for the overlap of genes downregulated by E2 at least 2-fold and derepressed by polyamide 1 at 0.3 or 1.0 µmol/L. C, hierarchical clustering (Euclidian distance, complete linkage) of genes changed at least 2-fold as compared with the induced state. D, 50 genes that were most changed by E2 induction were clustered (Eucludian distance, complete linkage). Of those genes, 30 were upregulated and 20 were downregulated by E2. Fold-changes are relative to E2-induced control.
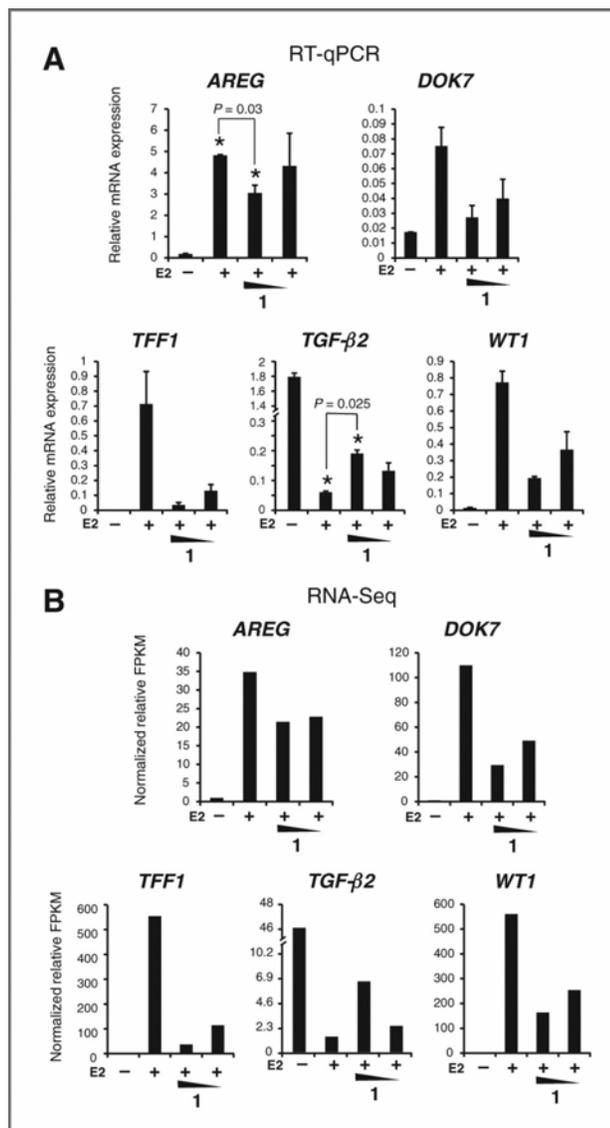
Figure 3.8 Confirmation of genome-wide polyamide effects observed by RNA-seq. Five genes (4 induced and 1 repressed by estrogen) were interrogated. A, relative mRNA levels of selected genes measured by qRT-PCR. B, relative mRNA expression values as measured by FPKM from RNA-seq. Concentrations of polyamide 1 are 1.0 and 0.3 μmol/L.
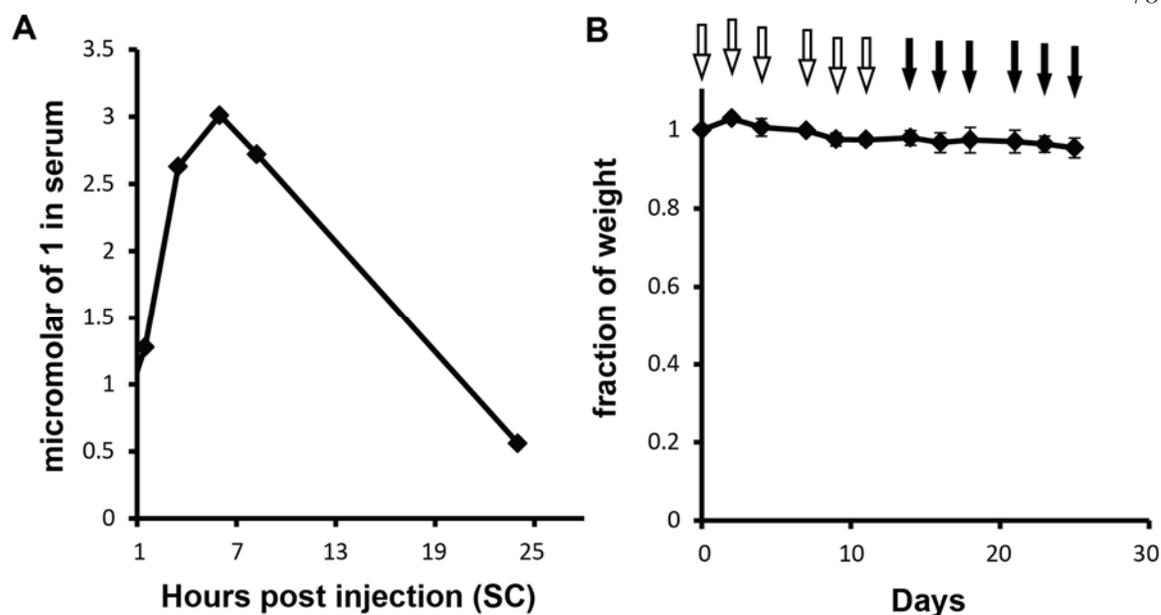
Figure 3.9 Pharmacokinetics of **1**. A, Serum concentrations of 1 after subcutaneous injection (120 nmol) into C57BL/6 mice. B, Weight curves after indicated injections of 1. Gray arrows: 20 nmol, Black arrows: 30 nmol. n = 4 mice.

Effects on ERα–mediated transcription in T47D-KBluc tumor bearing mice after short-term treatment

To measure the efficacy of polyamide 1 *in vivo* against E2-induced transcription, T47D-KBluc cells were engrafted into female nonobese diabetic/severe combined immunodeficient (NOD/SCID)-gamma (NSG) immunocompromised mice supplemented with a slow-release subcutaneous E2 pellet in the right flank to facilitate E2-induced growth. After 1 week of growth, mice were imaged using the IVIS Imaging System (Caliper) and stratified into groups of 12 mice each for vehicle and polyamide treatment. Polyamide 1 (25 nmol) in 200 μL 20% DMSO/PBS was injected subcutaneously into the left shoulder every other day for a total of 4 injections. Vehicle-treated mice received 20%

DMSO/PBS alone. After 3 injections, mice were reimaged. Luciferase output increased an average of 8-fold for the vehicle-treated mice and 3-fold for the mice treated with polyamide 1 (Fig. 3.10A). Mice were euthanized the day following the fourth injection for tumor resection. Tumors from vehicle-treated mice were 71 ± 12 mg and tumors from polyamide-treated mice 55 ± 11 mg (Fig. 3.10B), which does not explain the differences seen in luciferase expression. Representative images of mice treated with polyamide 1 or vehicle at day 6 are shown (Fig. 3.10C).

Effects on ERα–mediated transcription in T47D-KBluc tumor-bearing mice after long-term treatment

To investigate the effects of polyamide 1 in tumor-bearing mice after extended treatment, T47D-KBluc cells were again engrafted into female NSG mice supplemented with a subcutaneous E2 pellet in the right flank. Tumors were grown for 9 days before stratification of 5 mice each into polyamide 1 and vehicle treatment groups. Mice were treated with vehicle or 25 nmol of polyamide 1 in 20% DMSO/PBS, subcutaneously into the left shoulder twice a week for a course of 9 injections (25 days), beginning on day 16 after engraftment (Fig. 3.10D). Treated mice maintained their weights at more than 90% until the final days of treatment when their weights decreased to more than 85% before euthanasia. Luciferase was monitored weekly using the IVIS Imaging System. Luciferase output in the polyamide-treated mice was consistently lower than vehicle-treated mice (Fig. 3.10E). At the experimental endpoint, tumors from vehicle-treated mice were 165 ± 27 mg and tumors from polyamide-treated mice 128 ± 54 mg.
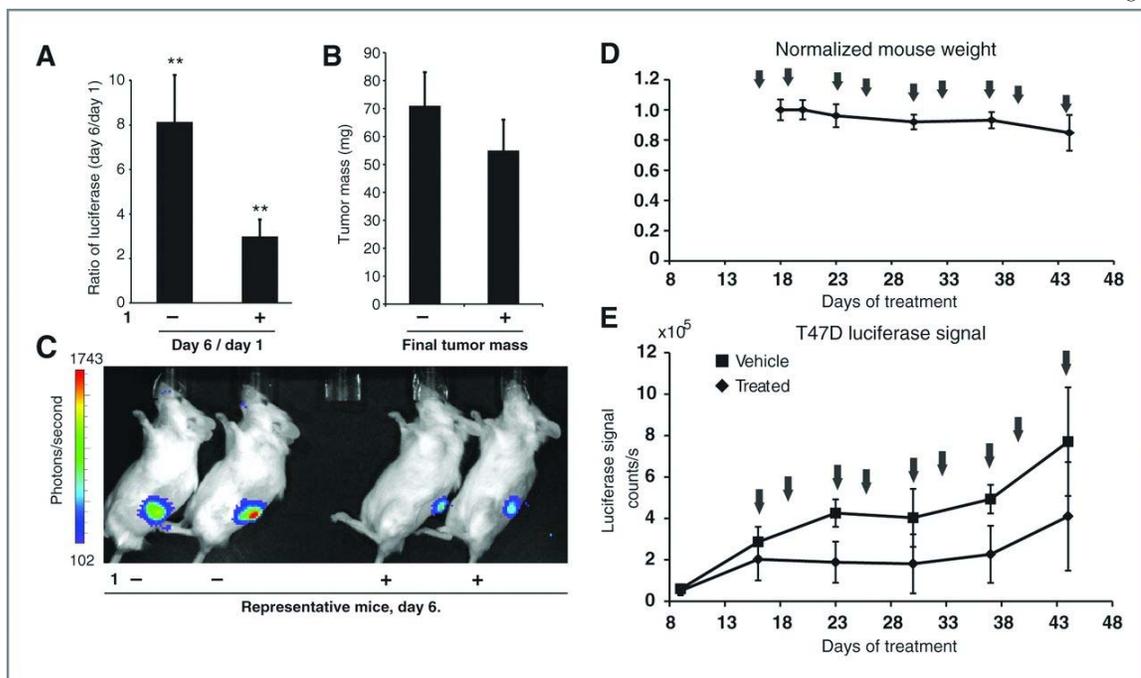
Figure 3.10 Xenograft studies. A, treatment of T47D-KBluc–bearing mice with polyamide 1 results in suppression of ER-driven luciferase. **, $P < 0.01$; $n = 12$ mice per group. Errors are 95% confidence interval (CI). B, tumor masses at experimental endpoint were vehicle: 71 ± 12 mg (95% CI); polyamide 1, 55 ± 11 mg (95% CI). C, representative luciferase output of vehicle and treated mice on day 6. D, treatment schedule for extended time-course experiments with normalized mouse weights over time. Arrows indicate treatment days. E, luciferase signal for polyamide 1 and vehicle-treated groups. Error bars are SDs. Mean tumor masses at the endpoint for animals treated with vehicle is 165 ± 27 mg (95% CI) and for animals treated with 1 is 128 ± 54 mg (95% CI).

Tissue distribution of FITC-conjugated polyamide 5 in mice bearing T47D-KBluc xenografts

Py–Im polyamide 5 is a FITC-labeled conjugate of hairpin **1** that was synthesized to evaluate tissue and subcellular localization via fluorescence microscopy. T47D-KBluc cells cultured *in vitro* and then treated with **5** showed nuclear fluorescence similar to what has been reported in other cell lines (27, 28) treated with FITC-conjugated polyamides in cell culture (Fig. 3.11). An NSG mouse engrafted with T47D-KBluc cells as described in the previous section was treated with polyamide **5** in a manner identical to that of polyamide 1, except at a dose of 50 nmol per injection. After 3 injections, the mouse was euthanized, the tumor resected, and internal organs dissected. Tissue was fixed, cryoprotected, sectioned,

and imaged immediately. Fluorescence signal was evenly distributed throughout multiple sections of the tumor xenograft. A representative section is shown (Fig. 3.12A). High magnification reveals nuclear localization in tumor tissue (Fig. 3.12B). Sections of cardiac muscle show significant cytoplasmic fluorescence in a fibrous pattern (Fig. 3.12C). Sections of kidney and liver both show nuclear fluorescence localization, with minimal cytoplasmic fluorescence (Fig. 3.12D and E). Small bowel epithelia show diffuse cellular fluorescence (Fig. 3.12F).
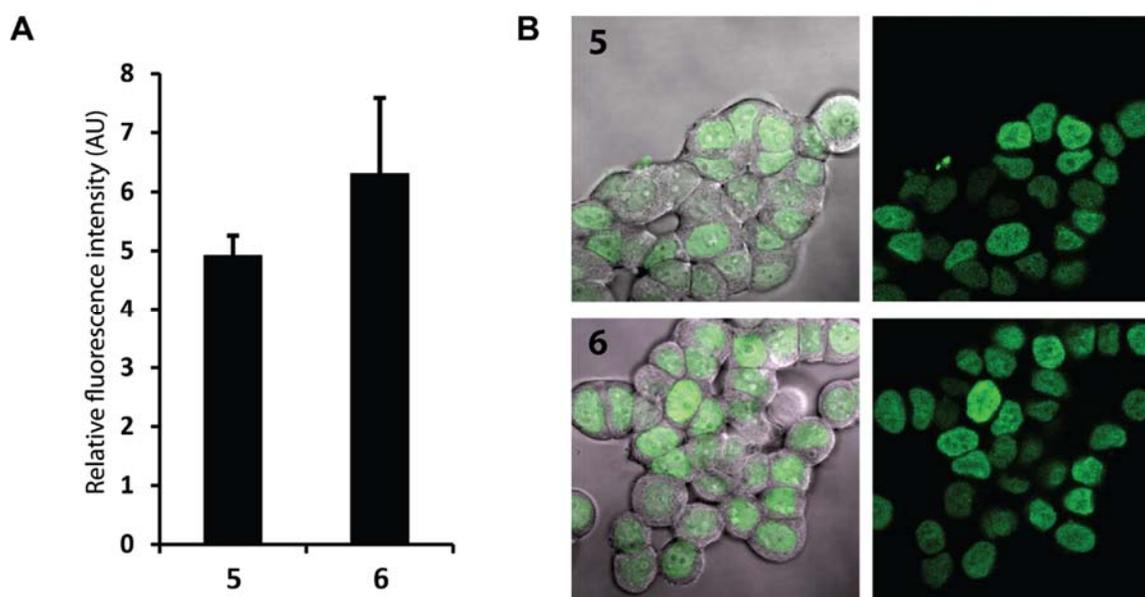
Figure 3.11. Confocal microscopy of live, cultured T47D-KBLUC cells. A, Mean intensity of fluorescence/cell averaged over three images. Errors are 95%CI. B, Representative images of polyamides 5 and 6.
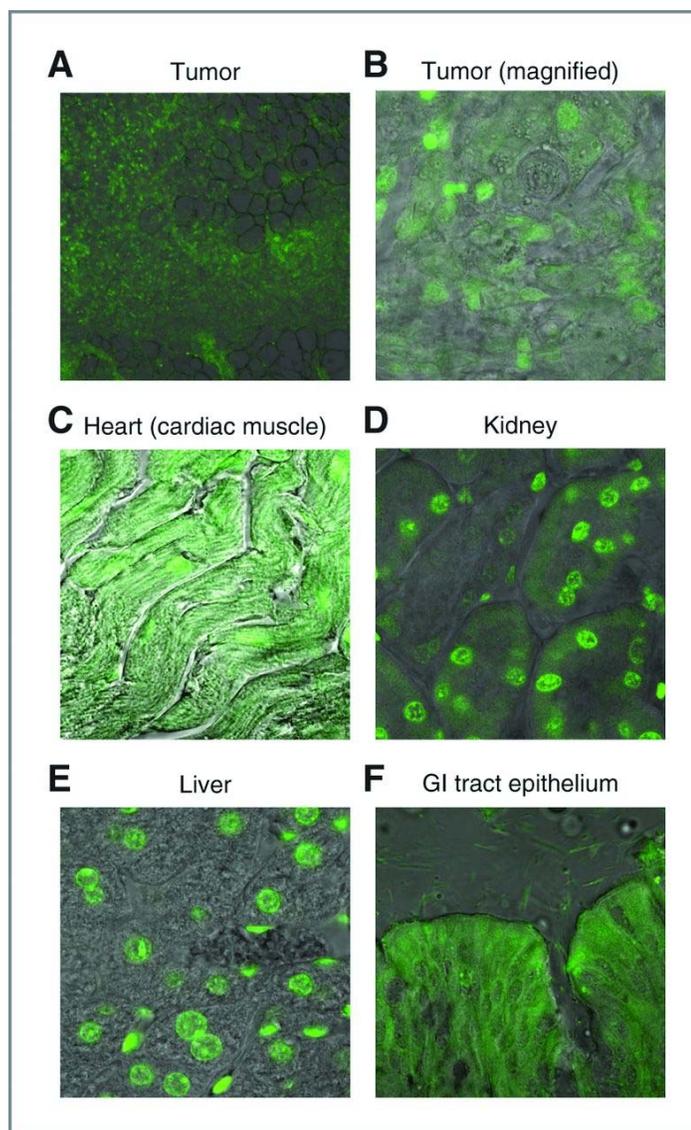
Figure 3.12 Tissue frozen sections of tissue extracted from xenograft-bearing mouse treated with polyamide 5. A, FITC-labeled Py–Im polyamide 5 distributes widely in sections of the T47D-KBluc tumor xenograft. B, high magnification shows nuclear localization of polyamide 5 in tumor cells. C, cardiac muscle sections show a fibrous pattern of fluorescence in the cytoplasm as well as nuclear staining. D, kidneys show nuclear localization of polyamide 5. E, liver sections show nuclear localization of polyamide 5. F, bowel epithelia show cytoplasmic fluorescence. GI, gastrointestinal.

To ensure that nuclear fluorescence in the xenografts was not an artifact of the fixation process, we extracted live cells from T47D-KBluc xenografted tumors from mice treated with polyamide 5 as earlier. In this experiment, cells were isolated via filtration and plated on microscope slides, and incubated for 6 hours before imaging. Cells derived from the tumor showed nuclear staining in a pattern similar to that seen in the fixed tumor sections as well as cultured cells treated with polyamide 5 *in vitro* (Fig. 3.13).
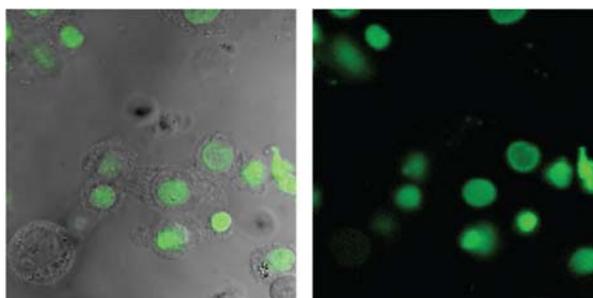
Figure 3.13 Confocal microscopy of live cells taken from T47D-KBLUC xenografts in mice treated with 5.

Discussion

In order for DNA-binding, Py–Im polyamides to be considered for therapeutic application, these molecules must possess favorable pharmacokinetic and pharmacodynamic properties and exert a desired effect in target tissues. In this study, ERα–induced transcription in xenografted breast cancer tumors was the target. A polyamide targeted to the ERE half site 5′-WGGWCW-3′ was identified from a focused screen for activity against ER-mediated transcription and cytotoxicity against ERα–positive breast cancer cells. This polyamide was further tested for its global effects on the transcriptome of E2-induced T47D-KBluc cells. Hairpin polyamide 1 showed limited toxicity and circulated at therapeutic levels in serum after subcutaneous injection. It also showed

activity against ER-driven luciferase expression in xenografted tumors in immunocompromised mice. FITC-polyamide conjugate 5 shows widespread localization in body tissues including sections through the xenografted tumor, which reveal nuclear fluorescence.

Suppression of ER-induced gene expression

We screened for both suppression of E2-induced luciferase expression and for antiproliferation by WST-1 assay using T47D-KBluc cells. Polyamide 1 was the most active by both measures, whereas polyamide 3 was inactive by either measure. These molecules differ only at a single atom, which represents the difference between a Py and Im heterocycle. Although polyamides have been shown to have differing uptake properties depending on Py–Im content and sequence (27, 28), the differences in activity in this series is likely not explained by differing uptake efficiency as confocal microscopy of FITC-polyamide conjugates 5 and 6 are similar (Fig. 3.11).

Global effects on the E2-stimulated transcriptome

E2 exerts its effects through direct DNA binding and less frequently extranuclear pathways that do not involve ERα–DNA binding (39). Our genome-wide transcriptome analysis (Fig. 4) revealed that a small fraction of gene expression changes induced by E2 treatment is suppressed by polyamide 1. From a total of 1,003 E2 upregulated genes, only 43 (0.43%) are repressed by polyamide 1 at 1.0 μmol/L, and from a total of 575 E2 downregulated genes, 95 (16.5%) were derepressed. However, among the 50 genes most strongly either induced or repressed by E2, a majority were significantly affected by polyamide 1. Among these top 50, 28 (56%) were downregulated by polyamide 1 (1.0

μmol/L) at least 2-fold. At a lower cutoff of 1.5-fold, 38 (76%) of E2-induced gene expression changes were abrogated by the action of polyamide 1 at 1.0 μmol/L. Many of these strongly E2-responsive genes play important roles in the development of tumors and are therapeutically relevant. Among them is Wilms tumor 1 (*WT1*), a gene originally identified as a tumor suppressor (40); however, more recently it has become apparent that it can also act as an oncogene (41). *WT1* expression is detectable in 90% of breast cancers (42) and high levels of *WT1* expression are correlated with poor patient survival (43). *TFF1* is a predictor for breast cancer patient survival (36). Transforming growth factor-β2 (*TGF-β2*) was observed among the most strongly E2-repressed genes and was also over 3-fold derepressed by polyamide 1 at 1.0 μmol/L. TGF-β2 is involved in cancer development that is also derepressed by traditional antiestrogens (44). We conclude that polyamide 1 acts in an antiestrogenic fashion among genes that are most potently affected by E2 but is less active for the majority of E2-responsive genes. If the mechanism by which polyamide 1 interferes with estrogen-driven gene expression is through direct interference with ER–DNA interfaces, we would not expect to affect ER-driven transcription at loci where ER signals through a tethering complex (45), such as with Ap1 and Sp1. Indirect interactions between ER and DNA through tethering with other proteins offer a partial explanation for the limited number of ER-driven transcripts affected by polyamide 1.

Most transcripts affected by polyamide 1 are not explained on the basis of E2 antagonism; 295 genes that are either up- or downregulated by polyamide 1, at least 2-fold that are not explained by effects on ER activity. Of these, 164 are upregulated and 131 downregulated by polyamide 1. To further characterize these effects, we used the DAVID functional annotation tool (46, 47). For the upregulated transcripts, enriched biologic

processes include those involved with the regulation of apoptosis and cell-death, as well as responses to endogenous and hormone stimuli, whereas downregulated genes suggested that polyamide 1 is involved in regulation of GTPase-mediated signal transduction and protein transport and biosynthesis (DAVID database, BP_FAT_GO analysis of genes changed at least 2-fold by treatment with 1 at 1 μM). The mechanisms of cell death may include the inhibition of transcription (10, 11, 13, 15), but other DNA-dependent processes may contribute and are an area of current investigation. Whether or not the effects of polyamide 1 on these biologic processes are specific to polyamide 1 or may represent a class-effect is unknown but also under study.

Polyamide treatment suppresses E2-simulated luciferase expression in vivo

T47D-KBluc cells were chosen as the cell line for our study based on previous work using T47D cells as a model for ERα–positive breast cancer. Both vehicle- and polyamide-treated groups showed an increase in total luciferase expression from the baseline measurement immediately before treatment on day 1. However, on day 6, after 3 sequential injections, this increase was significantly blunted in the polyamide-treated group as compared with vehicle (from ∼8- to 3-fold), suggesting that **1** was able to reach sufficient concentrations in tumor tissue to affect luciferase expression. The approximately 2.5-fold difference in luciferase between polyamide- and vehicle-treated groups, if interpolated to the *in vitro* data, suggest an approximate concentration of 0.3 μmol/L within the xenograft tissue. Tumor masses did not differ significantly between polyamide- and vehicle-treated mice in this experiment. However, this 6-day experiment may be too brief to adequately assess effects on tumor growth.

Effects on tumor size

Although there are no published reports on the growth of T47D-KBluc xenografts in mice, data from parental T47D xenografts show a slow, linear growth pattern rather than exponential (48). To better assay for antitumor activity of polyamide 1, we conducted similar experiments over a longer period of time. T47D-KBluc xenografted tumors were grown for 2 weeks before initiating treatment, and treatment with polyamide 1 was conducted twice per week for a total of 4 weeks. We observed no significant change in tumor size at the experimental endpoint, although we found a sustained suppression of luciferase output in the polyamide-treated arm as compared with vehicle-treated, consistent with our initial observations. The IC_{50} for cytotoxicity of polyamide 1 in cell culture is 0.47 µmol/L, which we believe to be higher than the concentrations achieved within the tumor tissues in this study.

Tissue distribution of FITC-conjugate polyamide 5 in mice after repeated subcutaneous injections

Fixed, frozen sections through multiple internal organs harvested from T47D-KBluc engrafted mice treated with polyamide 5 reveal widespread organ distribution of fluorescent signal but with differing patterns of fluorescence between tissues, and with little obvious systemic toxicity. The tumor sections show nuclear fluorescence in a subcellular pattern that is similar to what is observed in cell culture (Fig. 3.12A). The liver and kidneys also show strong nuclear fluorescence (Fig. 3.12D and E), whereas sections through the intestinal epithelium and cardiac muscle show predominantly cytoplasmic and both cytoplasmic and nuclear fluorescence, respectively. A difference in the cellular uptake

of polyamide–FITC conjugates between cell types has also been observed *in vitro* (27). Recent work has shown that polyamides can form aggregates in solution (49). Whether polyamide aggregation influences distribution *in vivo* is unknown. Tissue-specific targeting of small-molecule drugs is an area of current investigation that may become relevant for this class of molecules as additional *in vivo* experiments are planned.

Conclusion

Polyamide 1 delivered by subcutaneous injection in a simple DMSO/saline vehicle distributed widely in host and tumor tissue and showed adequate bioavailability to affect luciferase expression in xenografted tumor tissue, with acceptable toxicity. Future investigations will include optimization of polyamides for lower systemic toxicity without a compromise in efficacy.

Tables

Table 3.1 Genes induced (or repressed) by either **1** (1 µM) or E2 (10 nM)

Down >2-fold 1.0 µM PA

AC034193.1	FAF1	BCKDHB	PTPRK
GMDS	FTO	SERPINA1	PEMT
TFF1	LARS2	RP13-582O9.5	ERGIC1
RALGPS1	RSRC1	TTLL5	HIBADH
TRAPPC9	TBC1D22A	AIG1	WWP2
RP11-1018J8.2	ELP4	FARP2	NIPAL2
CTD-2313N18.7	CLSTN2	GRIK3	ADK
SMYD3	FAM172A	SDCCAG8	SND1
RP11-206M11.7	KIF16B	TNIK	C9orf46
SLC39A11	JPH2	ANO10	LHPP
U6.133	SLC24A3	LARGE	C3orf21
TFF3	CUX1	RABGAP1L	FOXJ1
EXOC4	C1orf194	MSI2	TYRP1
DENND1A	THSD4	SUCLG2	IQCK
AC126407.1	PCNXL2	PAPSS2	FLT4
IMMP2L	CBARA1	HINT1	MAPK9
C11orf49	COMMD10	MGAT5	ACOT7
TIAM1	CLPB	CCDC132	COBL
PDZK1P2	CASK	TSSC1	APBB2
PDZK1P1	RSU1	AC005077.9	PET112L
PDZK1	VPS53	MAPKAP1	KIFAP3
PIP	SIPA1L2	NT5DC1	DDX10
SERPINA6	ERC1	WDR70	KREMEN1
MAD1L1	PIGU	EFCAB11	OLFM1
SPINK4	FHL2	NME7	SLC6A14
ATG7	MYO1D	ERI3	METT5D1
CCDC91	RP11-40H20.1	AC006465.3	CHST1
DOK7	PIGL	APOD	GALNT2
AE000658.30	MGP	XPR1	PLCE1
CAPSL	CWC27	UQCC	OAZ3
PCCA	TSPAN5	STAU2	MNAT1
ACOX2	CDC20B	PCCB	THADA
WT1	FN1	IQCG	SLC38A6
SUSD3	NEK11	U6.694	NCRNA00263
RP11-161E22.2	RERG	OSGIN1	PKIG
VEPH1	EXOC6	PAM	C1QTNF6
MAP2K5	ITFG1	CRADD	ETNK2
ATG10	DEPTOR	OLFML3	TBC1D5
LRBA	AC023161.1	COPG2	NSMCE2
KIAA0391	DIP2C	RGS12	MRPL48
AC010859.1	BRE	ACER3	CRAT
ADAMTS15	RBM19	STK3	EXT2
COMMD1	MYEOV	EIF2B3	SERGEF
ARL15	TAB1	DTNB	

Up> 2-fold 1.0 µM PA

GABARAPL1	ZMIZ2	AC114498.3	AC044860.2
GABBR1	PABPC1L	CD44	AC121247.1
TROVE2	SLC6A9	CD74	RP11-539L10.3
SLC25A29	AMIGO2	snoU13.267	ANKRD10
GRB7	TUFT1	J01415.22	CASP4
LZTR1	PSD4	ADM	J01415.19
VEGFA	PNRC1	PLEKHB1	RND3
TNFAIP2	MICAL1	TP53INP1	J01415.4
SIRT1	HDAC10	MEGF6	RPS26P3
GALT	C3	TSPYL2	GPX3
HIST1H3D	PDP1	DUSP1	RNASEH2B
LYPD3	RARRES3	KLHL24	HIST1H2AD
DHX58	TXNIP	TP53I11	AL593851.1
PCDH1	RPL12P4	SLC3A2	SCARNA17
PPAPDC1B	LPIN3	MUTYH	ATHL1
ENGASE	PLXNA1	ALDH2	MB
HES1	OGT	MX1	J01415.1
MDM4	PTPRU	MTUS1	AL355388.1
HIST1H2BD	RPL12P8	5S_rRNA.486	AC092143.1
RP5-857K21.14	RP3-405J24.1	RP11-466C23.3	RC3H1
NFKBIA	ABCC5	J01415.20	AQP3
C9orf3	OGFRL1	JDP2	AC136698.1
CCNG2	RP11-154D6.1	J01415.24	MTRNR2L9
IFRD1	CALD1	GPRC5A	J01415.5
AC011737.2	RP11-601I15.1	SELENBP1	J01415.8
IL1R1	AC022007.5	J01415.15	J01415.6
ZNF251	OPLAH	IFI27	snoU13.458
BAMBI	AL132821.1	RP4-697K14.7	Y_RNA.184
CC2D1B	RP11-373E16.1	hsa-mir-1977.1	TGFB2
PABPC1P10	HIST2H4B	J01415.10	TNFAIP3
ZNF302	HIST2H4A	CBS	UPK2
PARP10	RALGDS	CDC42EP3	ARRDC3
AMOTL2	MOSC1	SERTAD2	ELF3
GIGYF1	AGA	IFI6	RPL12P1
RP11-169K16.6	CLK4	RP11-383C5.4	J01415.2
OAS1	RP11-106M3.1	U6.1189	GDF15
ESPN	FAM193B	J01415.18	snoU2_19.4
BX322557.10	OSR2	ASNS	AL132988.3
RP11-434O22.1	AP005264.1	EP400NL	5_8S_rRNA.6
RP11-344H11.4	TMEM44	J01415.7	S100P
RCN1	AC064874.2	MTRNR2L2	RP5-1174J21.1
SOX4	NDRG1	RASL11B	AC005261.1
SLC7A5	AC010487.1	MGEA5	SERGEF
ISG15	CTSL1	COL6A1	

Up >4-fold 1.0 µM PA

TGFB2	ELF3	snoU2_19.4	RP5-1174J21.1
TNFAIP3	RPL12P1	AL132988.3	AC005261.1
UPK2	J01415.2	5_8S_rRNA.6	
ARRDC3	GDF15	S100P	

E2 >4-fold up

RP11-459E5.1	RP11-21L23.3	NDC80	KIF11
SPINK4	TRIP13	SPAG5	U6.694
WT1	AMZ1	HMMR	KIFC1
TFF1	RBP7	WNT10B	SLC7A1
PDZK1P2	LAT2	NBL1	C1orf97
RP11-1018J8.2	FAM65C	AC026271.3	RP11-467L20.6
DOK7	AGR3	TSKU	KIF20A
PDZK1P1	EXO1	MELK	TNFRSF10A
PDZK1	DERA	TOP2A	AZGP1
ACOX2	RP11-467L13.5	SKAP2	CCNB1
STC2	RRM2	SIPA1	CDCA2
AREGB	CCNA2	MTHFD2	TUBA1B
AREG	MND1	CDCA8	AC097711.1
OLFM1	KCTD6	DLGAP5	FAM72A
TFF3	SPC25	FAM83D	NUSAP1
AC135050.3	CDCA3	MYBL2	MCM2
SLC6A14	JPH2	HJURP	PRC1
MYEOV	DSCC1	ESPL1	TK1
PTGES	NCAPH	GINS1	TPX2
CXCL12	MKI67	U6.664	KIAA1524
TNS4	BIRC5	DTL	CCND1
SUSD3	MGP	UHRF1	HINT1
NKAIN1	CENPA	TNFRSF10C	OIP5
TP53I3	AFF3	RBM24	UBE2T
FLT4	AGR2	CDKN3	C9orf100
LY6E	THBS1	GNG11	C6orf141
PEG10	DEPDC1B	CDC20P1	FANCI
EIF4E1B	CDC6	CDC45	RP4-774G10.1
LIN28A	PLK1	ASF1B	XPR1
PFKFB3	KIAA0101	SNCG	GPSM2
GRIK3	CMTM7	KIF4A	CCDC85B
NMU	SULF1	RP11-67L3.6	RP11-379F12.3
RAPGEFL1	PRR11	ADCY3	SERPINA6
SYT12	CDCA5	LMNB1	PCNA
NCAPG	RRM2P3	CKAP2L	C15orf23
CA8	NUF2	AURKA	PCK2
GREB1	KIF2C	WDR76	DEPDC1
FHL2	THSD4	PRIM1	MZT2B
AC126407.1	UBE2C	CASC5	AMD1
LDHB	CENPH	TACC3	CRLF1
IGFBP4	TYMS	CTPS	OAS3
MYC	BUB1	AL592284.1	ARHGAP11A
NPY1R	AC006465.4	HMGB2	RP11-336N8.4
AL358781.2	ABCC11	RP11-424C20.2	RPL7AP11
RBBP8	SLC29A1	SLC16A3	RP11-120E5.5
MICB	TROAP	RP11-145M9.3	FEN1
PTTG1	CCNB2	CDC20	RP5-1100H13.3
AURKB	RERG	SH3BP1	SNX24
GALNT7	PKMYT1	BARD1	CKS2
SPC24	CEP55	NRM	NEK2
MCM10	BUB1B	CA12	RP11-417L14.1

E2 >4-fold down

CDH10	C9orf117	AP001816.1	FGFR1
AC005261.1	CDKN2B	AMT	HIST1H2AC
UPK2	CITED2	MALAT1	PYROXD2
CDHR3	MAP2	AC114498.8	ENC1
TGFB2	AC114498.3	CAPSL	J01415.12
AQP3	CCDC159	AC114498.4	FSTL1
snoU13.267	RP5-1174J21.1	PDP1	MAFK
5_8S_rRNA.6	C11orf66	NAALADL2	PLXNB1
SOD3	EPAS1	RP11-612B6.2	CALCOCO1
ZDHHC8P1	ANXA3	CD24P4	SLC25A29
AL132988.3	PNPLA7	O5R2	ID2
RP11-383C5.4	DUSP1	HIST1H2AI	AC093734.1
ARL4D	RP11-400K9.4	LCN12	SELENBP1
GPNMB	SOX2	MYO1B	TBC1D9
VTCN1	CALD1	AMIGO2	U6.133
RASL11B	CSAD	AL713999.8	HIST1H1PS1
5S_rRNA.486	HPX	KCNMA1	ERBB2
BCAS1	EPGN	LRRC46	CCDC39
CCDC154	snoU2_19.4	COL7A1	AC060834.2
NDRG1	GNMT	snoU13.458	ARRDC3
CHST1	BAMBI	ARMC3	TUFT1
CDC42EP3	TXNIP	AC064874.2	THBS3
TRIM29	HIST1H2AD	CPAMD8	HIST1H4J
hsa-mir-1977.1	J01415.11	AC136698.1	BTG1
NANOS1	IL1R1	ARHGEF37	TP53INP1
ELF3	HES1	RP4-717J23.3	GSTM2P1
HIST1H4H	J01415.9	C1orf194	MLLT4
U6.1189	ID3	MTERFD3	HIST1H3D
J01415.10	AL132988.2	RP11-539L10.3	PIK3R3
FGFR4	L1CAM	KIAA1984	FAM134B

Down >4-fold 1.0 µM PA

AC034193.1	SLC39A11	PDZK1P2
GMDS	U6.133	PDZK1P1
TFF1	TFF3	PDZK1
RALGPS1	EXOC4	PIP
TRAPPC9	DENND1A	
RP11-1018J8.2	AC126407.1	
CTD-2313N18.7	IMMP2L	
SMYD3	C11orf49	
RP11-206M11.7	TIAM1	

Table 3.2 Genes whose induction (or repression) by E2 is inhibited
(or repressed by) **1 (1 μM)**

Genes upregulated by E2 >2-fold and downregulated by 1 >2-fold

SPINK4
ACOX2
OLFM1
SLC6A14
FLT4
GRIK3
FHL2
JPH2
MGP
RERG
HINT1
ACER3
AC034193.1
CLSTN2
APOD
MAPK9
OSGIN1
C9orf46
TAB1
ETNK2
AC005077.9
ACOT7
OLFML3
RP13-582O9.5
SERPINA1
VEPH1
EIF2B3
DDX10
WT1
TFF1
PDZK1P2
RP11-1018J8.2
DOK7
PDZK1P1
PDZK1
TFF3

MYEOV
SUSD3
AC126407.1
THSD4
U6.694
SERPINA6
RP11-206M11.7

Genes downregulated by E2 >2-fold and upregulated by 1 >2-fold

TGFB2
snoU13.267
5S_rRNA.486
NDRG1
hsa-mir-1977.1
J01415.10
AC114498.3
DUSP1
CALD1
BAMBI
TXNIP
IL1R1
HES1
PDP1
OSR2
AMIGO2
AC064874.2
SLC25A29
SELENBP1
TUFT1
TP53INP1
HIST1H3D
RP11-466C23.3
OPLAH
GPRC5A
MEGF6
SOX4

J01415.4
PSD4
AC022007.5
CCNG2
HIST2H4A
HIST2H4B
TMEM44
MB
ZNF251
AC010487.1
LYPD3
PNRC1
MTUS1
HIST1H2BD
LPIN3
MTRNR2L2
GABBR1
ABCC5
BX322557.10
KLHL24
CLK4
GABARAPL1
FAM193B
GRB7
RP5-857K21.14
AC044860.2
ANKRD10
C9orf3
SCARNA17
PLXNA1
JDP2
HDAC10
PABPC1L
AMOTL2
ZMIZ2
LZTR1
ESPN
RC3H1
J01415.19
TSPYL2
ENGASE

PPAPDC1B
J01415.24
AC005261.1
UPK2
AQP3
5_8S_rRNA.6
AL132988.3
RP11-383C5.4
RASL11B
CDC42EP3
ELF3
U6.1189
RP5-1174J21.1
snoU2_19.4
HIST1H2AD
snoU13.4S8
AC136698.1
RP11-539L10.3
ARRDC3
MTRNR2L9
J01415.1
AL593851.1
RALGDS
PTPRU
ATHL1
CC2D1B
CASP4

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