Chapter 5

Py-Im polyamides inhibit DNA Topoisomerase II activity in vitro by disrupting enzyme-DNA binding

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Abstract

We have developed 8-ring, hairpin, Py-Im polyamides that downregulate the expression of Androgen Receptor (AR) target genes in LNCaP cells. These compounds are cell-permeable, sequence-specific, high-affinity DNA minor groove binders that are designed to prefer the sequence 5'-WGWWCW-3' (W=A or T). In this study, we investigate the interaction of these two compounds with topoisomerase II (Top2) isozymes using *in vitro* and cell culture techniques. We determine the polyamides to inhibit Top2 catalytic activity by preventing enzyme-DNA binding. However, the DU145 cell culture results in Top2 isozyme knockdown constructs are more consistent with Top2 poisons. Further investigation using flow cytometric analysis of cell cycle distribution indicates that polyamide treatment results in S-phase arrest, a finding that is not associated with Top2 inhibitors or Top2 poisons. The possibility that polyamides are acting as DNA-damage inducing agents or as DNA synthesis inhibitors is discussed.

5.1. Introduction

Pyrrole-imidazole polyamides are programmable, sequence-specific, high-affinity DNA minor groove binders (1). Using pairwise configurations of N-methylpyrrole (Im) and N-methylimidazole (Im) amino acid monomers arranged in antiparallel fashion in a hairpin configuration, they can be designed to bind specifically to a wide array of DNA sequences using a simple set of pairing rules (2). An Im-Py pair recognizes G-C, a Py-Im pair recognizes C-G, and a Py-Py pair is degenerate for A-T or T-A (W) (3). Due to steric constraints, hairpin polyamides have additional binding preferences for an A-T or T-A base pair at either side of the core set of heterocycles; therefore an 8-ring hairpin polyamide specifies a 6 base pair binding site (4). In addition to their DNA binding properties, polyamides permeate cells, localize to the nucleus, and bind chromatin without the need for delivery agents (5,6).

When combined with affinity and sequence specificity approaching that of endogenous transcription factors, these favorable biocompatibility properties have made it possible to use polyamides as tools for establishing chemical control of gene expression. Polyamides designed to target the consensus DNA-binding sequences of endogenous transcription factors have been used to disrupt the genotropic actions of those proteins. By blocking transcription factor-response element binding, polyamides can abrogate activation of target gene expression. This technology has been used successfully in cancer cell tissue culture to target the transcription factors HIF1a, androgen receptor (AR), glucocorticoid receptor (GR), and AP-1 (7-10). In each case, genomic occupancy of the target transcription factor at relevant loci was decreased approximately 2-fold as measured by chromatin immunoprecipitation (ChIP) when cells were treated with an appropriately targeted polyamide.

As DNA minor groove binders, polyamides would be predicted to have a wide range of effects on DNA-dependent cellular processes (11). In addition to their effects on

transcription factor binding and inhibition of target gene induction, interaction with other biological processes could be contributing to the observed changes in gene expression (12). Minor groove binders are known to inhibit the DNA topoisomerases Top1 and Top2, as well as DNA helicases (13-18). Inhibition of any one of these enzymes could contribute to non-specific transcriptional inhibition. Yet data from cDNA microarray analyses of polyamide-treated cells suggest that the resultant mRNA inhibition is not a simple global downregulation (7,8,19). In fact, the magnitude and number of transcriptional changes produced by the polyamide used to antagonize AR and GR activity are similar to those produced by other small molecule, non-DNA binding antagonists of those pathways.

Top1 and the two isozymes of Top2, Top2α and Top2β, are known to be intimately involved in gene transcription in mammalian cells (20). The strand separation required to accommodate the RNA Polymerase II holoenzyme produces superhelical strain in the form of positive supercoiling in advance of the transcription bubble (21). Top1 and Top2α are associated with this complex and are required to enable it to advance (22); inhibition of either of these two enzymes would be expected to produce a global decrease in transcription. The isozymes of Top2 are highly homologous and differ chiefly in their regulation; Top2α expression is correlated with the cell cycle, Top2β is not (23). Yet inhibition of Top2β activity would produce a more limited response, as activity of this isozyme is only required for expression of a subset of regulated genes (24). This subset corresponds to the target genes of several nuclear hormone receptors, including AR and GR (24-26). The interaction of polyamides with the two Top2 isoenzymes is the chief subject of this study.

To define the interaction of polyamides with Top2, we chose to study two AR-targeted polyamides, compounds **1** and **2** (Figure 5.1). Using *in vitro* relaxation assays with a purified Top2α fragment, we demonstrate that both compounds displayed dose-dependent inhibition of topoisomerase activity without forming a cleavable complex. Further analysis with a titration of increasing amounts of Top2α suggested that the inhibition was due to blocking Top2 binding to DNA. Cell culture assays using a Top2α shRNA-

knockdown cell line conferred significant resistance to polyamide-induced cytotoxicity. We then investigated the effects of polyamide treatment on the cell cycle to look for the characteristic G_2/M arrest produced by Top2 inhibitors and Top2 poisons. Unexpectedly, we observed S-phase arrest in both the wild-type and Top2α knockdown cell line, which suggests that the primary effects of polyamides are unrelated to Top2.

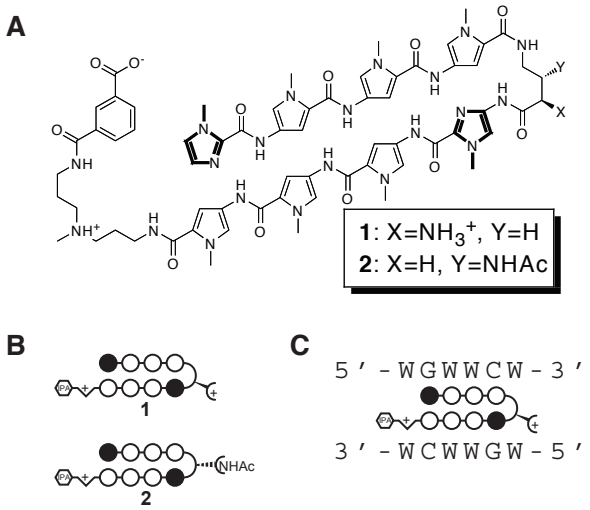

Figure 5.1. Chemical structure and binding preferences of the Py-Im polyamides used in this study. (A) Chemical structure showing the two different turn functionalities. (B) Ball-and-stick representation of the polyamides. Open circles represent N-methylpyrrole residues, filled circles represent N-methylimidazoles. The hexagon represents the isophthalic acid moiety. (C) Both 1 and 2 bind the same 5'-WGWWCW-3' DNA sequence, where W = A or T.

5.2. Results

5.2.1. Polyamides inhibit Top2 catalysis by preventing the enzyme from binding DNA.

Top2-targeted small molecules generally fall into two categories: inhibitors of enzymatic activity and topoisomerase poisons (27). Inhibitors prevent the enzyme from catalyzing the DNA strand breakage, either by inhibiting enzyme binding or by inhibiting the ATP hydrolysis Top2 requires to perform its catalytic cycle. They do not produce DNA damage. Topoisomerase poisons trap the enzyme in a covalent complex with the DNA, stabilizing the otherwise transient double-strand DNA break. Further interactions of this complex with cellular proteins quickly result in DNA damage. Minor groove DNA-binding compounds are predicted to inhibit topoisomerases by preventing enzyme binding.

To look for polyamide-mediated inhibition of Top2, we conducted *in vitro* relaxation assays using supercoiled pHOT1 plasmid DNA and a purified Top2α fragment (Figure 5.2). The

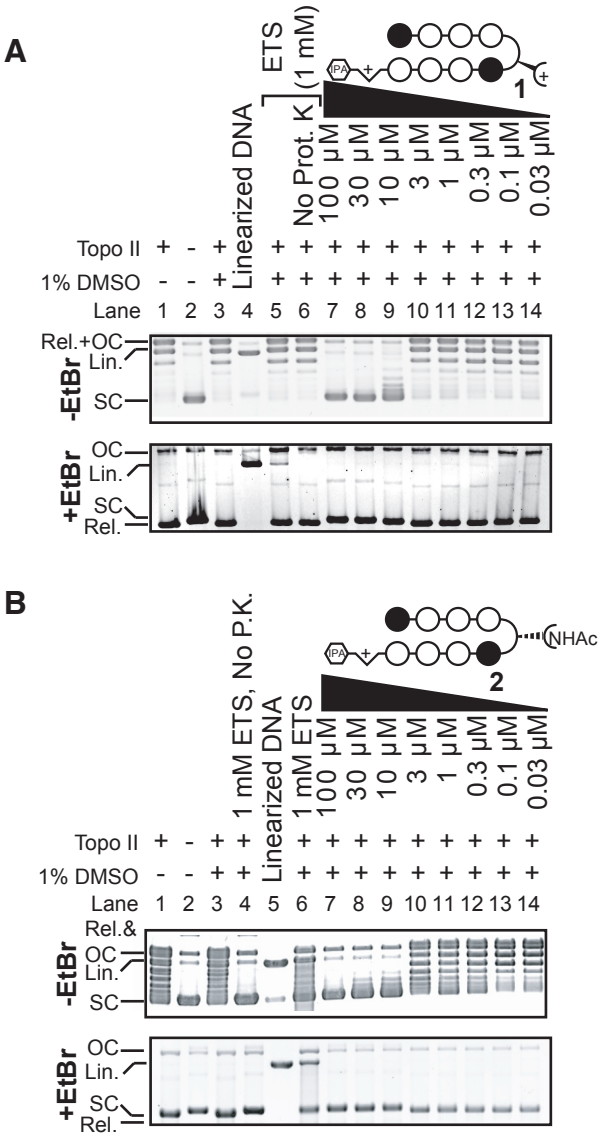

Figure 5.2. *In vitro* DNA relaxation assay demonstrating polyamide-mediated, dose-dependent inhibition of Top2α-p170 catalytic activity without cleavage complex formation. Results of agarose gel electrophoretic analysis of supercoiled pHOT1 plasmid DNA relaxation by Top2α-p170 run in the absence (top) or presence (bottom) of 0.5 µg/mL ethidium bromide (EtBr). (A) Results obtained by adding increasing concentrations of compound **1** (0.03-100 µM) to relaxation reactions. (B) Results obtained by adding increasing concentrations of compound **2** (0.03-100 µM) to relaxation reactions.

reaction products were analyzed by agarose gel electrophoresis after quenching, proteinase digestion, and phenol-chloroform extraction. To look for inhibition of relaxation, the gels were run in the absence of ethidium bromide (EtBr) (Figure 5.2a and 5.2b, upper panel) and post-stained with SYBR-Gold; supercoiled substrates have a smaller relative size and migrate faster on the gel. The linearized, relaxed, and open-circular forms of the plasmid have similar apparent size and tend to migrate together (compare Lanes 3 and 4, Figure 5.2a, upper panel). Etoposide was included as a positive control for cleavage complex formation (Figure 5.2a, Lane 5; Figure 5.2b, Lane 6, upper panel). In the presence of ATP, both polyamides inhibited plasmid relaxation in a dose-dependent manner (Figure 5.2a and 5.2b, upper panel, lanes 7-14).

Samples from these reactions were also run in gels containing EtBr to look for formation of a cleavage product. In these gels, the unwinding effect of the intercalative dye dominates the topology; all closed circular forms of DNA will be positively supercoiled and will migrate with similar R_f. This allows unambiguous identification of the linearized DNA that forms when a small molecule causes Top2 cleavage complex formation (compares Lanes 3 and 4, Figure 5.2a). Etoposide-treated samples and linearized DNA were included as positive controls for cleavage complex formation (lane 5, Figure 5.2a, lower panel and lane 6, Figure 5.2b, lower panel). An etoposide-treated, proteinase K-free sample was also included to demonstrate detection of

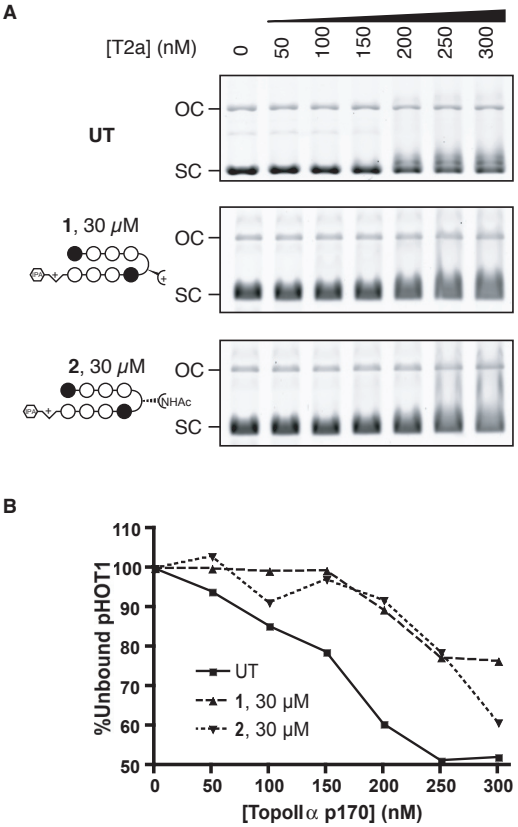

Figure 5.3. Polyamides **1** & **2** inhibit Top2α-p170 binding *in vitro*. (A) Gel electrophoretic mobility shift assay for Top2α-p170 DNA-binding to pHOT1 in the presence or absence of 30 μM **1** or 30 μM **2**. The amount of free DNA decreases as the protein is titrated up to 300 nM. No ATP is present in this assay. (B) Top2α-p170 DNA-binding curves based on relative quantitation of (A).

the covalent complex. Failure to digest the protein resulted in extraction of the covalent complex into the phenol-chloroform layer (compare lanes 5 and 6, Figure 5.2a, lower panel). Neither polyamide caused cleavage complex formation over a wide range of concentrations (Lanes 7-14, Figures 5.2a and Figure 5.2b, lower panel).

Enzymatic inhibition in the absence of cleavage complex formation is consistent with a mechanism based on disruption of Top2 binding. To address this mechanism more directly, we treated samples of pHOT1 plasmid DNA with 30 μM concentrations of **1** or **2** for 30 minutes and then added increasing amounts of Top2α in the absence of ATP (Figure 5.3). Enzyme-DNA binding was measured by gel-shift assay. Treatment with 30 μM of either compound increased the amount of free DNA per microgram of Top2α added. Compound **1** was more potent at inhibiting Top2-DNA binding than compound **2**.

5.2.2. Topo2a knockdown confers apparent resistance to polyamide-mediated cytotoxicity. The expression level of Top2 isozymes is known to modulate the cytotoxicity of agents targeted to these enzymes (28,29). As polyamides inhibit Top2 activity *in vitro* by inhibiting enzyme binding, decreasing Top2 expression would be expected to sensitize cells to their cytotoxic effects. On the other hand, if polyamides were acting as Top2 poisons despite the *in vitro* data, Top2 knockdown would be expected to confer resistance to their cytotoxicity, as decreased Top2 expression would produce fewer of the lethal DNA double-strand breaks that result from cleavage complexes.

To look for a correlation between Top2 isozyme expression and polyamide cytotoxicity, we determined cytotoxicity IC_{50} values for compounds **1** and **2** in DU145 prostate cancer cells and three derivative cell lines containing stably incorporated shRNA constructs: DU145-shT2α, DU145-shT2β, and DU145-shCntrl. The first two contain Top2α- and Top2β-targeted shRNAs, and the third is included as a scrambled vector control (30). The polyamides demonstrated dose-dependent inhibition of cell growth in each cell line (Figure 5.4a-b). The data also show that Top2α knockdown confers approximately

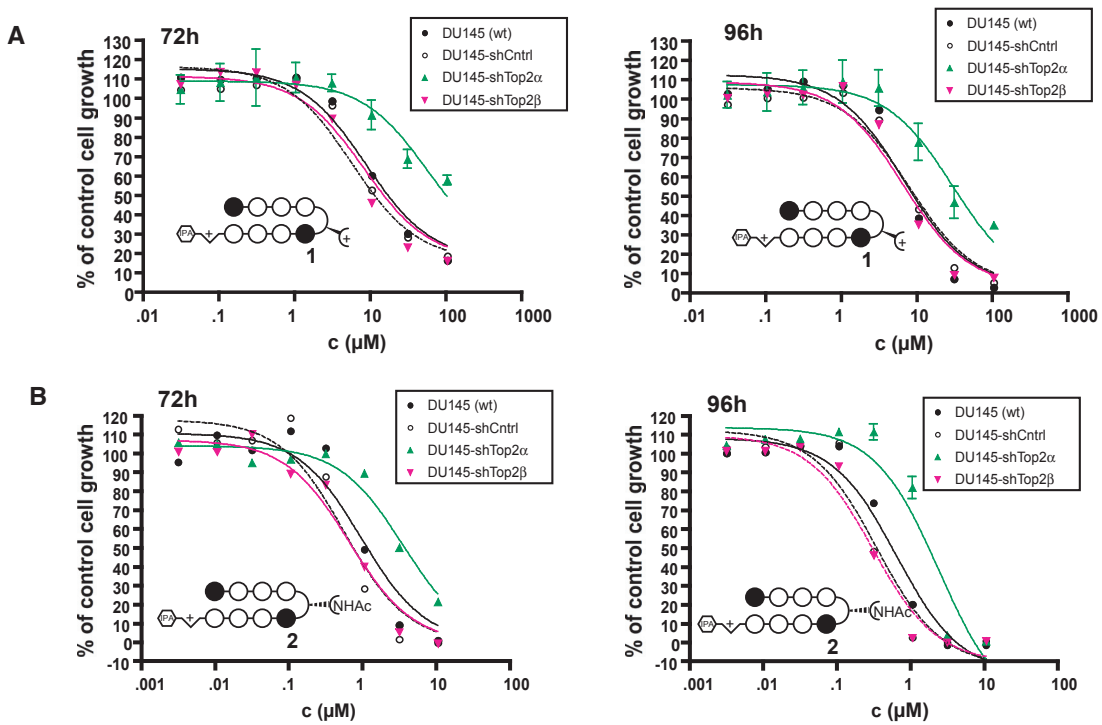

Figure 5.4. Dose-dependent induction of cytotoxicity by polyamides **1** and **2** in DU145 (wt) and Top2 knockdown cell lines. Dose-response curves generated by sulfarhodamine B staining of DU145 (filled circles), DU145-shCntrl (open circles), DU145-shTop2α (green triangles), or DU145-shTop2β cells (pink triangles) treated with **1** (A) or **2** (B) for 72 or 96 h.

5-fold resistance to polyamide treatment for cells treated for 72 or 96 h (Table 5.1), which is similar to the resistance conferred on known cleavage-complex producing agents like etoposide (30). These results would appear to contradict the *in vitro* data that suggest that polyamides inhibit enzyme-DNA binding and do not cause cleavage complex formation.

5.2.3. Polyamide treatment causes S-phase arrest. Top2 inhibitors and Top2 poisons are

Compound	Time (h)	DU145 (wt)	DU145-shCntrl	DU145-shTIIα	DU145-shTIIβ
1	72	14±4	13±5	90±30 **(6)**	9±1
	96	8±4	10±5	40±10 **(5)**	7±2
2	72	1.5±0.2	1.1±0.1	8±1 **(5)**	1.1±0.1
	96	0.76±0.06	0.7±0.1	2.5±0.6 **(3)**	0.6±0.1

Table 5.1. Cytotoxicity IC_{50} values (μM) of compounds **1** and **2** in DU145 and DU145-shTop2 cell lines. The data are determined from non-linear least squares analysis and are shown as the mean ± s.d. of three independent biological replicates. The fold resistance conferred to polyamide-mediated cytotoxicity by Top2α knockdown is shown in bold.

known to produce cell cycle arrest in G_2/M. The former inhibit decatenation of replicated chromosomes during mitosis, and the latter activate DNA damage repair pathways at the G_2/M checkpoint (31). To look for phenotypic evidence consistent with polyamide-mediated inhibition of Top2 suggested by the *in vitro* studies and the cytotoxicity results, we performed a flow cytometric cell cycle assay. We subjected DU145 cells to treatment with various concentrations of **1** and **2** for 24 hours before pulse-labeling them with EdU for absolute S-phase analysis by flow cytometry (Figure 5.5a). Fixed cells were labeled for EdU incorporation using an AlexaFluor dye and then co-stained for total DNA content with 7-aminoactinomycin D to display the entire cell cycle. Contrary to our expectations, both compounds produced a dose-dependent increase in the number of cells in S-phase, not G_2/M. Gemcitabine, a DNA synthesis inhibitor, was included as a positive control for S-phase disturbance.

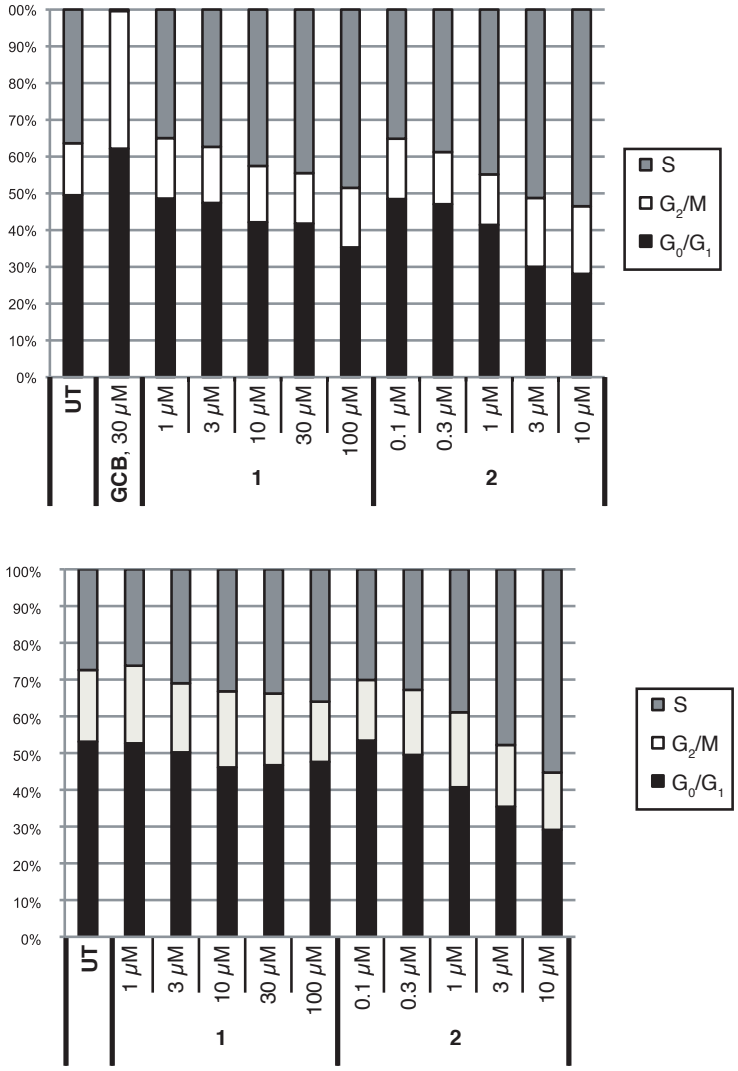

Figure 4.5. Polyamide treatment causes S-phase arrest in DU145 and DU145-shTop2a cells. (A) Cell cycle distribution of DU145 cells treated with compounds or controls for 24h as measured by two-color flow cytometric evaluation of A488-EdU pulse-labeled cells stained for DNA content with 7AAD. Gemcitabine is included as a control for a known DNA synthesis inhibitor. Both compounds increase the percentage of cells in S-phase in a dose-dependent manner. (B) Analogous experiment conducted in DU145-shTop2α cells showing a lower number of S-phase cells in the untreated condition but a similar dose-dependent increase in the S-phase population for both compounds. 20,000 cells were counted for each sample. The experiment was conducted in duplicate.

We then performed a

similar experiment in the Top2α-knockdown cell line that showed resistance to polyamide-induced cytotoxicity. The polyamides displayed similar dose-dependent induction of S-phase arrest to that observed in the parental cell line (Figure 5.5b). The disturbance was detected over the same concentration range. When compared to the results in the parental cell line, the percentage of DU145-shT2α cells in S-phase was lower, but this was also true in the untreated controls. The percentage of cells in G_2/M was higher than in the parental DU145 cells. This pattern is consistent with slowed transit through G_2/M due to decreased Top2α and the absolute requirement for this enzyme to perform decatenation prior to mitosis.

5.3. Discussion

The *in vitro* relaxation assay data appear to conflict with the results obtained in the Top2-isozyme knockdown cell lines. The former demonstrate that polyamides **1** and **2** inhibit Top2 activity by preventing enzyme-DNA binding, which agrees with the results observed for the related compound, distamycin A (32). The polyamides inhibit Top2 binding and enzymatic activity at micromolar concentrations in a rank order according to their relative affinities for DNA (Figure 5.3). Neither compound shows any evidence of creating the cleavable complex that leads to DNA double-strand breaks. On the other hand, the cytotoxicity IC_{50} values are consistent with a compound that does create Top2α-mediated DNA damage (Figure 5.4), although this time the relative potencies are reversed (Table 5.1). This apparent contradiction prompted further investigation of the cellular response to polyamide treatment in an attempt to provide phenotypic evidence to support one of the two competing hypotheses.

Surprisingly, the results of the cell cycle investigation did not produce the G_2/M-phase arrest that usually accompanies Top2α inhibition or poisoning. Instead, they unambiguously demonstrate that compounds **1** and **2** produce S-phase arrest. The arrest is both dose dependent and relatively rapid compared to the 72 or 96 h timescale of the cytotoxicity results. The two compounds showed the same pattern of potency as in the cytotoxicity assays, with **2** being more potent and producing greater S-phase arrest than **1**. The observed cell cycle disruption due to polyamide treatment is not consistent with a Top2α inhibitor or a Top2α poison; S-phase arrest is characteristic of compounds that produce S-phase specific DNA damage or inhibition of DNA replication (33).

In addition, the cell cycle pattern observed in the untreated condition in the DU145-shT2α cell line suggests an explanation for its relative resistance to polyamide-mediated cytotoxicity. This cell line showed a higher percentage of cells in G_2/M, and a lower percentage in S-phase, which is likely due to decreased Top2α availability for decatenation. If polyamides were indeed acting as S-phase-specific toxins, then having fewer cells in S-

phase at any given time would produce fewer cytotoxic interactions.

Taken together, these data suggest that Top2 inhibition is not a primary effect of polyamide treatment in cell culture. The *in vitro* data would appear to conflict with the cytotoxicity results in the knockdown models, and the S-phase interaction is not phenotypically consistent with Top2 inhibition. S-phase arrest is usually due to either S-phase specific DNA damage, or inhibition of DNA synthesis (33). It is possible that the polyamides are inhibiting Topoisomerase I, which is highly active during S-phase (20). Treatment with the Top1 poison camptothecin does cause DNA damage as the replication holoenzyme encounters the cleavable complex (27). But as non-reactive DNA minor groove binding ligands, polyamides would not be expected to form adducts with DNA. However, it is possible that cellular metabolism could transform the compound into a reactive species. The polyamides could also be directly impeding fork progression, perhaps by inhibiting a replicative helicase; distamycin is known to inhibit the DNA damage repair helicases WRN and BLM *in vitro* (34). Further investigation of the S-phase arrest produced by polyamide treatment should look for polyamide-induced DNA damage. Elucidation of the cell signaling responsible for producing the S-phase delay could also be informative; activation of the ATM/ATR kinases would signal checkpoint activation in response to DNA damage or replicative stress (33,35). If no DNA damage is detected, *in vitro* replication assays could provide further support to a helicase-based mechanism of DNA synthesis inhibition.

5.4. Materials and methods

5.4.1. Chemicals and reagents. Compounds **1** and **2** were synthesized on solid phase Kaiser oxime resin using previously published protocols (36). Cell culture media was purchased from Invitrogen, and fetal bovine serum from Irvine Scientific. Gemcitabine was purchased from AvaScientific. Etoposide was purchased from Sigma-Aldrich, as were all other reagents unless otherwise noted.

5.4.2. *In vitro* DNA relaxation and Top2α-DNA binding assays. Materials for these assays were supplied by Topogen (Port Orange, FL). For relaxation assays, 540 ng Top2α-p170 fragment (16 units) was added to 250 ng supercoiled pHOT1 DNA in assay buffer (0.05 M Tris-HCl (pH 8), 0.15 M NaCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol) plus 2 mM ATP with or without test compounds in a total volume of 20 μL. The dimethylsulfoxide (DMSO) concentration was standardized to 1% for all samples except the no-DMSO solvent controls. Reactions were incubated at 37 °C for 30 min. and then quenched with 2 μL 10% sodium dodecyl sulfate solution. Samples were then extracted with chloroform: isoamyl alcohol 24:1, mixed with 2 μL 10x glycerol loading buffer and loaded onto 1% agarose gels in tris-acetic acid-EDTA (TAE) buffer with or without 0.5 μg/mL ethidium bromide (EtBr). Gels run without EtBr were post-stained with SYBR-Gold (Invitrogen). For Top2α-DNA binding assays, increasing amounts of Top2α-p170 fragment were added to aliquots of 0.325 μg supercoiled pHOT1 plasmid DNA in assay buffer plus 2.5% glycerol with or without test compounds. Samples were incubated at 37 °C without ATP for 30 minutes and then loaded directly onto 1% agarose gels, run in TAE buffer, and then post-stained with 1x SYBR-Gold.

5.4.3. Cell culture. DU145 cells were purchased from ATCC (Manassas, VA) and maintained in RPMI 1640 with 10% FBS at 37 °C under 5% CO_2. DU145-shCntrl, DU145-shT2α,

and DU145-shTβ2 cells were a gift from R. Dorr at Arizona Cancer Center (Tucson, AZ) and were maintained in RPMI1640/10% FBS supplemented with 0.5 μg/ml puromycin to maintain selection for the knockdown constructs (30).

5.4.4. 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 (37). Cells were plated at 2,000 or 2,500 cells per well. Compounds were added in 100 μL 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 μL 10% trichloroacetic acid solution, washed, stained, and dried as described. After solubilization of the bound dye in 10 mM Tris (pH 8), the absorbance was measured at 490 nm on a Victor microplate reader (PerkinElmer).

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^((Log EC50-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.

5.4.5. Cell cycle analysis. 800,000 cells were plated in 10 cm diameter dishes for 24 h before treatment with test compounds for an additional 24 h. 10 μM EdU was added 30 min before harvest. The cells were harvested by trypsinization and combined with the cell culture supernatant before pelleting at 300 x g. Following overnight fixation in 70% ethanol, the cells were rehydrated in 1% BSA/PBS and processed with the Click-it EdU Alexa Fluor 488 Flow Cytometry assay kit (Invitrogen) using half the recommended A488 reagent. After overnight treatment with 0.2 mg/mL RNase A in 1% BSA/PBS, the cells were stained for DNA content with the provided 7-aminoactinomycin D and analyzed on

a FACSCalibur (Becton-Dickinson) instrument. The data were analyzed using FlowJo v8.8.2 (TreeStar) and are representative of two trials.

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