Chapter 3

Py-Im Polyamides Inhibit DNA Replication in Cell-Free Extracts from X. laevis Oocytes

This research was conducted in collaboration with Joon Lee, William G. Dunphy, and Peter B. Dervan (California Institute of Technology).

Abstract

The effects of DNA-binding pyrrole-imidazole (Py-Im) polyamides on DNA replication in *Xenopus* oocyte cell-free extracts were investigated for the purpose of elucidating the mechanism of polyamide-induced replication stress. Inhibition of nucleotide incorporation was observed in this system in response to multiple polyamides targeting different 6 base pair DNA sequences, consistent with previous results found in mammalian cells. Disruption of the pre-replication complex (pre-RC) was probed as a potential cause of inhibition; however, the pre-RC factors Orc2 and MCM2 as well as helicase-associated Cdc45 were all recruited to chromatin in polyamide treated extracts. Activation of the ATR checkpoint was investigated for evidence of replication fork stalling, as observed in mammalian cells. Polyamide treatment did not result in phosphorylation of Chk1 or MCM2, suggesting that the ATR checkpoint was not activated and that an early step in the cell cycle was likely inhibited. Staining of sperm chromatin incubated in polyamide treated extracts revealed that chromatin decondensation was inhibited, preventing replication initiation. Comparison to other small molecule DNA binders suggests that inhibition of chromatin decondensation is likely due to impaired nuclear membrane formation. These results show that inhibition of chromatin decondensation should be studied as a possible effect of polyamides in mammalian cells, and that a different model system should be employed to study the effects of polyamides on active replication forks.

3.1 Introduction

The previous chapter detailed the evidence for polyamide-induced replication stress in an asynchronous population of DU145 human prostate carcinoma cells (1). This low-level stress was sufficient to activate ATR but not the downstream effector kinase Chk1. In order to investigate the generality of these effects more cell lines should be tested, as each cancer cell line harbors unique genetic alterations that can affect major signaling pathways. Another method for testing the generality of polyamide-induced replication stress is to test for polyamide effects *in vitro* using cell-free extracts that are capable of undergoing cell cycle progression including DNA replication and DNA damage response signaling. Cell extracts are beneficial because they can be easily synchronized and arrested, thus allowing the polyamide to be dosed before induction of the cell cycle in a controlled manner. Cell extracts also allow for direct comparison of different sequences of polyamides and accurate measurements of effective concentrations, given that there is no variability due to uptake across cell membranes. The cell extract system most frequently employed to study the cell cycle and DNA replication is from *Xenopus laevis* oocytes.

Oocyte extracts from *X. laevis*, the African clawed frog, are made by harvesting healthy eggs, packing them, and then crushing them by ultracentrifugation. After centrifugation, the middle layer containing the cytosol and membranes is then separated from the top layer containing lipids and the bottom layer containing the dark yolk and pigments (2). Xenopus oocyte extracts produced by this relatively low speed centrifugation are arrested at metaphase of meiosis II and will undergo nuclear membrane

formation and DNA replication upon the addition of demembraned sperm nuclei and exogenous calcium for release from cytostatic factor (CSF) arrest. The use of sperm chromatin provides a large concentrated and diverse sequence of DNA targets for the polyamide to interact with, similar to experiments in cell culture. Importantly, no transcription occurs during the first cell divisions, and therefore polyamide effects on transcription as opposed to processes related to DNA replication can be reasonably ruled out. These egg extracts contain a high concentration of the molecular machinery, regulatory factors, checkpoint proteins, and damage response proteins necessary to execute eukaryotic cell division. Egg extracts can be used to monitor the loading of specific replication factors to determine which specific step in replication is inhibited and monitor any DNA checkpoint signaling that might occur in response to replication stress. It also allows for individual factors to be easily depleted through the use of antibodies in order to probe for the functional roles of specific replication and stress response factors. Another important benefit of this system is the wealth of data on the effects of common replication inhibitors for comparison against polyamides. This was an issue previously, as the DU145 cell line is not commonly used to investigate DNA replication.

In this study we report that Py-Im polyamides inhibit nucleotide incorporation in frog egg extracts. We also show that inhibition of DNA replication is not the result of impaired loading of pre-replication complex (pre-RC) factors, and that polyamide treatment does not induce phosphorylation of either Chk1 or MCM2, which suggested a lack of ATR signaling activation. Finally we show that polyamides prevent proper decondensation of sperm chromatin, the DNA template in this system, causing inhibition

of DNA replication initiation. These results suggest that while DNA replication is inhibited in this model system, the mechanism is likely different from what we observed in DU145 given the difference in DNA checkpoint response signaling. However, the effect on chromatin decondensation is interesting, and represents another potential effect of polyamides in mammalian cells.

3.2 Results

Py-Im polyamides inhibit DNA replication in Xenopus egg extracts.

The most general method for testing replication inhibition by polyamides is to measure the extent of nucleotide incorporation in activated extracts. DNA replication was probed using alpha phosphate-substituted ^{32}P-dATP ([α-^{32}P]-dATP). Xenopus oocyte extracts mixed with sperm chromatin were incubated with increasing concentrations of polyamide **1**, which targets the 6 base pair sequence 5'-WGWWCW-3'. Treatment with **1** resulted in decreased dATP incorporation, with full inhibition of nucleotide incorporation occurring between 1 and 2.5 μM. This result was consistent with observations in DU145 cells measuring the incorporation of EdU (Figure 3.1).

Using cell-free extracts also allows for direct comparison of polyamide targeted to different sequences. We therefore compared the effects of three different polyamides, as well as two other DNA-binding small molecule, actinomycin D and distamycin A, and the DNA polymerase alpha inhibitor aphidicolin. Polyamide **2** is targeted to the sequence 5'-WGGWCW-3' and polyamide **3** is targeted to 5'-WGGWWW-3'. The polyamides and distamycin were all dosed at 500 nM while aphidicolin and actinomycin were dosed

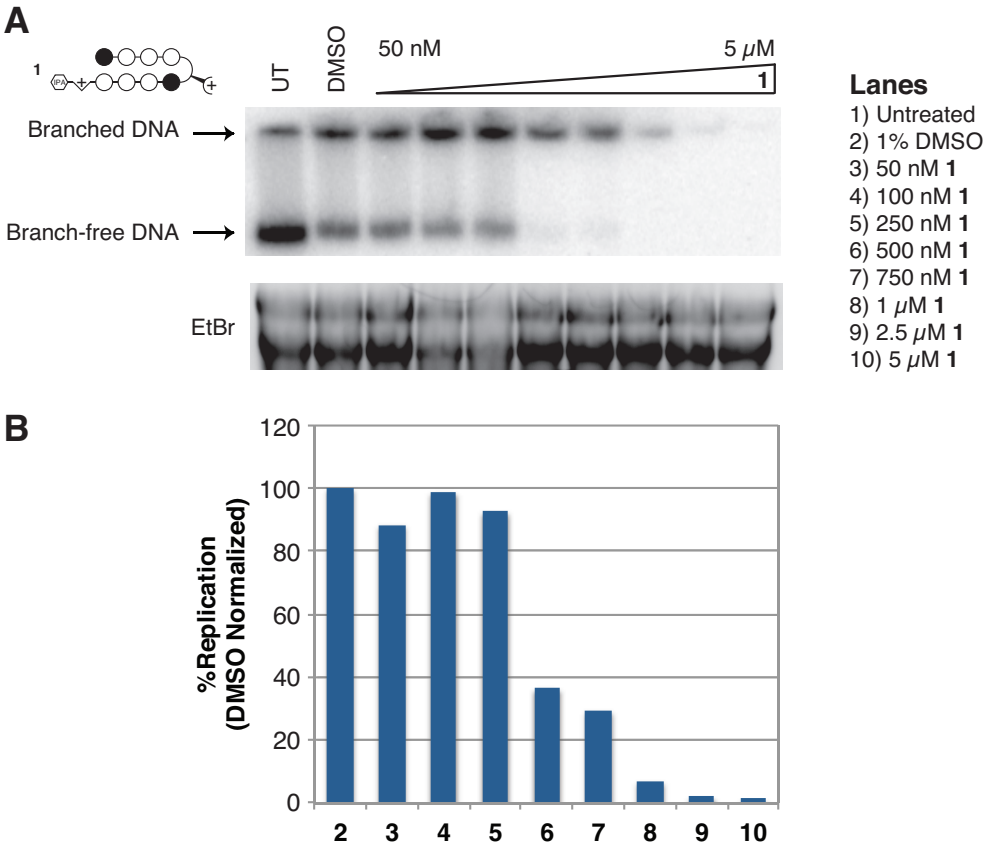

Figure 3.1 Polyamide **1** inhibits DNA replication in *Xenopus* oocyte extracts. (**A**) Frog egg extracts arrested at Meiosis II containing 3000 sperm nuclei/μL were treated with increasing concentrations of **1** (50 nM – 5 μM) just prior to release from CSF arrest by 0.4 mM $CaCl_2$. After 100 min, an aliquot of each sample was run out on an agarose gel, which was then dried and visualized on a storage phosphor screen. Total DNA content in the extracts was estimated by ethidium bromide staining of the gel. Polyamide **1** is shown by ball-and-stick representation. It targets the 6 bp sequence 5'-WGWWCW-3'. (**B**) The percent of DNA replication completed was estimated by measuring the band intensity for the entire lane and shown in the bar graphs in the bottom panel. The IC_{50} was ~500 nM, and maximal inhibition was observed at 2.5 μM. Data is representative of two replicates.

at 10 ng/μL. Significant inhibition of nucleotide incorporation was observed for all compounds tested with the exception of distamycin, which showed only weak inhibition (Figure 3.2). Weaker inhibition by distamycin is consistent with the lower binding affinity of distamycin to DNA compared to Py-Im polyamides.

Py-Im polyamide treatment does not inhibit loading of pre-Replication Complex factors.

The previous results showed that DNA replication is inhibited in Xenopus egg extracts. Next, we wanted to investigate which step might be inhibited. We first tested whether treatment with Py-Im polyamides affects loading of the pre-replication complex, which consists of Cdt1, Cdc6, and the origin recognition complex (ORC) as well as the MCM2-7 helicase. The ORC proteins, composed of Orc1-6, in addition to Cdt1 and Cdc6 are responsible for loading two MCM2-7 hexameric helicases onto the DNA prior to replication (3). In order to test whether polyamides inhibit loading of the pre-RC onto DNA, the replicated oocyte extract was fractionated, and purified chromatin was collected. Next, the proteins were separated by SDS-PAGE and immunoblots were run against representative pre-RC factors, Orc2 and MCM2. We also probed for Cdc45, which is another component of the replicative helicase with the MCMs. In addition, we probed for RPA70, which is the large subunit of the single stranded DNA (ssDNA)-binding protein replication protein A (RPA) and accumulates on chromatin under replication stress.

For this experiment, we included a non-activated control (lane 1), which lacked both calcium and sperm chromatin. These conditions showed no significant detection of Orc2, MCM2, Cdc45, or RPA (Figure 3.3A) as expected. Two positive controls, which inhibit the loading of different pre-RC factors, were also tested for comparison. Geminin negatively regulates the cell cycle by inhibition of Cdt1, thus preventing loading of the MCMs (4). Geminin-treated extracts showed loading of Orc2, but not MCM2 or associated Cdc45 (lane 6). The protein p27, which inhibits cyclin E-CDK2, thus

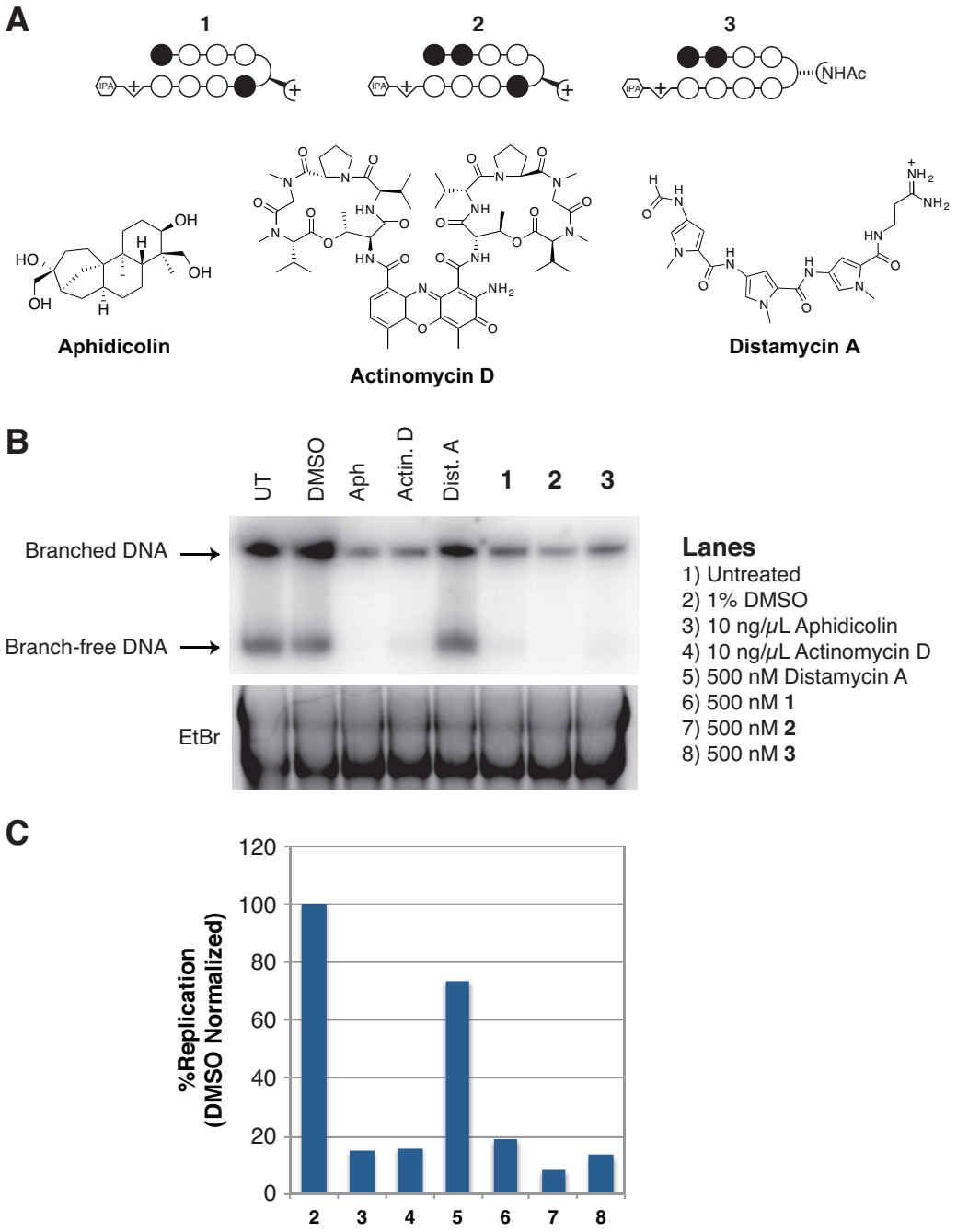

Figure 3.2 Py-Im Polyamides targeted to different DNA sequences equally inhibit DNA replication in *Xenopus* oocyte extracts. (**A**) Ball-and-stick representations of polyamides and chemical structures of other inhibitors used in this experiment. (**B**) Depiction of the extent of [α-^{32}P]-dATP incorporation from egg extracts treated with a variety DNA-binding small molecules, including aphidicolin (Aph), actinomycin D (Actin D), distamycin A (Dist A), and polyamides **1**-**3**. (**C**) The percent of DNA replication completed was estimated by measuring the band intensity for the entire lane and shown in the bar graphs in the bottom panel. At the concentrations tested, DNA replication was significantly inhibited by all of the tested small molecules except distamycin A. Data is representative of two replicates.

preventing downstream loading of Cdc45, was also tested. Extracts treated with p27 showed loading of Orc2 and MCM2, but not Cdc45 (lane 5). Aphidicolin-treated extracts were included as a representative inhibitor of active DNA replication that induces replication fork stalling and ssDNA accumulation. These extracts showed loading of MCM2, as well as weak loading of Cdc45 and Orc2 (lane 4). In addition, aphidicolin treatment induced significant loading of RPA70, confirming ssDNA accumulation. Extracts treated with 1 μM **1** showed significant loading of Orc2, Cdc45, and MCM2, but not RPA70 (lane 3). Extracts treated with DMSO showed similar results (lane 2). Therefore, no aspect of pre-RC loading was affected by polyamide treatment. In addition, polyamide treatment did not cause significant ssDNA formation as observed under treatment with aphidicolin.

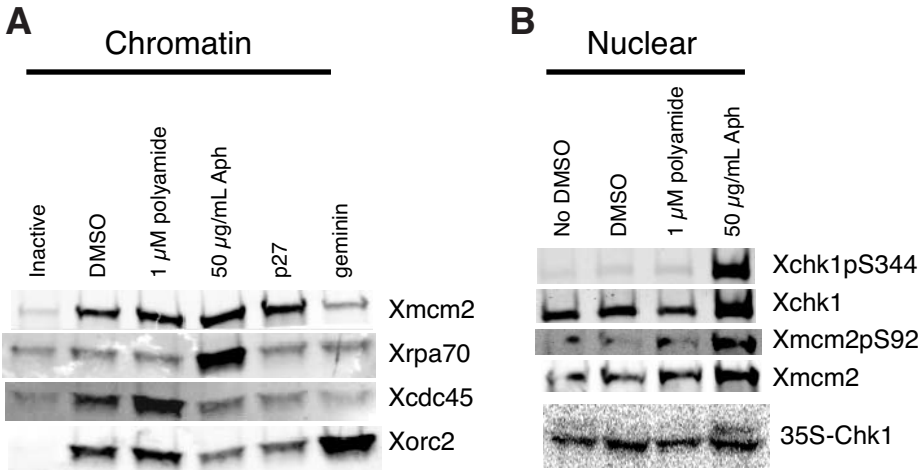

Figure 3.3 Polyamide **1** does not inhibit loading of pre-RC factors or activate ATR checkpoint signaling. (**A**) Chromatin loading of representative pre-RC factors Orc2 and MCM2, as well as the helicase component Cdc45 and the large subunit of ssDNA-binding protein RPA70 were probed by immunoblot. Extracts were untreated or treated with 1% DMSO, or **1**, as well as aphidicolin, p27, and geminin for comparison. Polyamide treatment did not inhibit pre-RC loading or result in the accumulation of ssDNA. (**B**) Nuclear lysates were prepared from untreated extracts and extracts treated with DMSO, **1**, or aphidicolin and probed for phosphorylation of Chk1 and MCM2 by immunoblot. Only treatment with aphidicolin resulted in phosphorylation of MCM2 and Chk1, and therefore **1** does not activate ATR checkpoint signaling. Blots are representative of two replicates.

Py-Im Polyamide treatment does not induce phosphorylation of ATR targets.

The previous experiment showed that loading of pre-RC factors was not inhibited by polyamide treatment. We next investigated whether polyamide treatment affected active replication. In order to test this, we probed for the activation of the ATR-checkpoint pathway given that partial activation of the pathway was observed in response to polyamide treatment in DU145 cells (Chapter 2). Chk1 S344 (corresponds to human Chk1 S345) and MCM2 S92 (corresponds to human MCM2 S108) phosphorylation were chosen as representative targets of ATR. Chk1 phosphorylation was readily observed in extracts treated with aphidicolin, but not polyamide **1** (Figure 3.3B). This result was consistent with observations in DU145 cells (Chapter 2). The lack of Chk1 phosphorylation was also confirmed using 35S-Chk1. A slower migrating band representing the heavier phosphorylated form of the protein was only observed in extracts treated with aphidicolin. Interestingly, MCM2 S92 was also not phosphorylated in response to polyamide treatment, despite being phosphorylated in DU145 cells. Given the lack of phosphorylation observed for both targets, it is unlikely that polyamide treatment induces ATR activation. Therefore, polyamide-induced inhibition of replication likely acts through a different mechanism than in DU145 cells.

Py-Im polyamide treatment inhibits chromatin decondensation.

Inhibition of DNA replication without affecting loading of the pre-RC complex or activating the ATR checkpoint pathway, suggested that a process prior to these steps might be inhibited by polyamide treatment. Sperm chromatin from male frogs used for this system is initially in a condensed form. Upon activation of the extracts, the DNA

undergoes decondensation via a rapid nucleoplasmin-dependent step and a slower nuclear membrane-dependent step (5). Only after DNA decondensation and formation of a functional nuclear membrane can replication begin. Previous studies on polyamide:DNA binding showed that these molecules can bind to DNA wrapped around in histones in a nucleosome core particle, demonstrating the potential for polyamides to interact with condensed DNA (6). Therefore, we investigated whether polyamide **1** was inhibiting sperm chromatin decondensation.

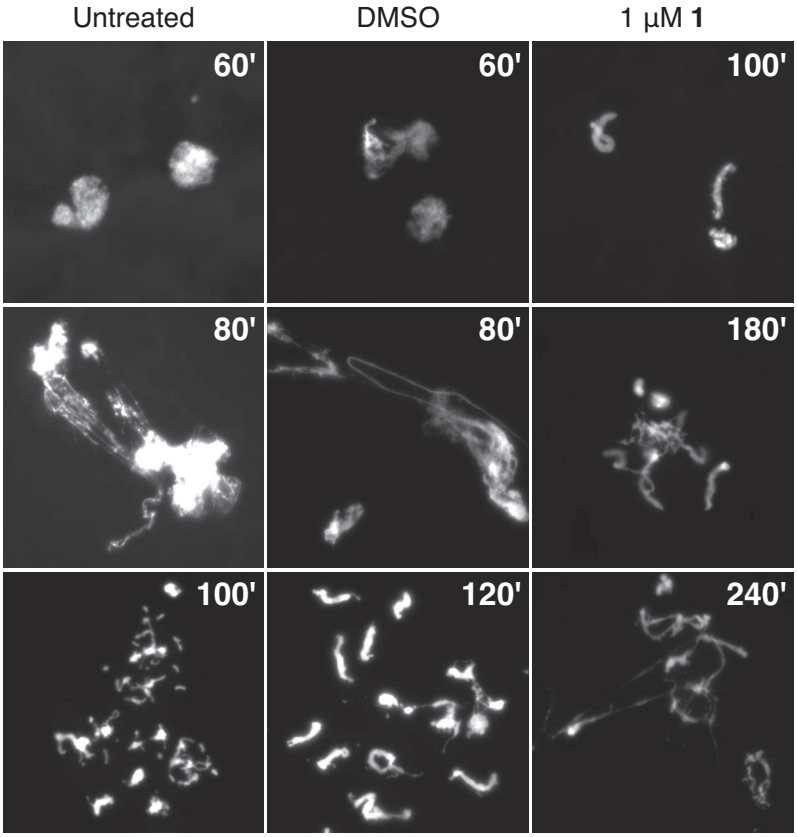

Figure 3.4 Polyamide **1** inhibits decondensation of sperm chromatin. Aliquots of activated untreated extracts and extracts treated with 1% DMSO or **1** were taken at the indicated time point at the DNA stained with Hoechst 33258. The cell cycle was completed by 100 min in untreated lysates, which is consistent with previous studies. DMSO slowed down the cell cycle slightly; however, there was no obvious defects. Treatment with polyamide **1** results in inhibition of sperm chromatin decondensation, which dramatically slowed down the cell cycle. Images are representative of two replicates.

In order to monitor this step, extracts are activated as in other experiments, but aliquots are removed at regular time intervals for visualization by staining with Hoechst 33258. After 60 min incubation, nearly all chromatin in the untreated extract is decondensed and contained in fully formed nuclei. By 80 min, the DNA in untreated cells entered telophase (Figure 3.4). Finally, at 100 min nuclear envelope breakdown had occurred and DNA condensed into chromosomes, ready for another cycle. DMSO-treated extracts completed all of the same steps, though 120 min was required to complete a single cell cycle. Extracts treated with 1 μM polyamide **1**, however, showed striking differences from the untreated and DMSO-treated extracts. The sperm chromatin in extracts treated with **1** remained in a condensed state even after 100 min. By 180 min, some DNA appeared to be in a decondensed state similar to telophase. Finally, at 240 min, most of the DNA was decondensed but it had not recondensed as expected at the completion of the cell cycle. These results demonstrated that polyamide treatment prevents proper decondensation of sperm chromatin, which can prevent replication from ever initiating.

Previous efforts investigating plausible mechanisms for replication inhibition *in vitro* demonstrated that polyamides are capable of inhibiting DNA helicase activity. However, polyamide-induced inhibition of sperm chromatin decondensation occurs prior to DNA replication initiation, and therefore it is unclear whether polyamides can inhibit active replication forks. In order to test this hypothesis using the low speed supernatant extracts, polyamides need to be dosed at times after release from CSF arrest in order to bypass the initial chromatin decondensation and nuclear membrane formation steps. For

this experiment, extracts mixed with sperm chromatin were activated with $CaCl_2$ followed by the addition of polyamide **1** 15 or 30 min later in order to allow time for nuclei to form and DNA to decondense. Reduced incorporation of [α-^{32}P]-dATP was observed at both time points, though the effect was much weaker when polyamide was added after 30 min compared to 15 min or when the compound is added prior to release from CSF-arrest (Figure 3.5). These results suggest that it is likely that polyamides can inhibit actively replicating forks, albeit weakly. However, under these conditions, the total incubation time of polyamide with DNA is sufficiently short that the polyamide and DNA were not fully equilibrated.

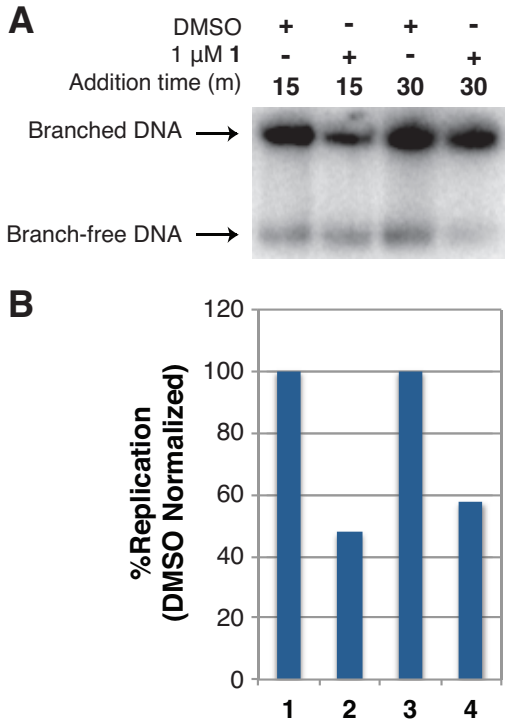

Figure 3.5 Polyamide **1** inhibits DNA replication when dosed after DNA decondensation step. (**A**) Depiction of the extent of [α-^{32}P]-dATP incorporation from egg extracts treated with DMSO or 1 μM **1** either 15 or 30 min after release from CSF arrest by $CaCl_2$. (**B**) Bar graphs showing the extent of nucleotide incorporation. At the time points tested, DNA replication was inhibited by polyamide **1**, although the effect observed was less than when polyamide was dosed prior to the addition of $CaCl_2$. Data is representative of two replicates.

3.3 Discussion

In this study we demonstrated that Py-Im polyamides targeted to a variety of 6 base pair DNA sequences are all equally capable of inhibiting DNA replication in the cell-free *Xenopus* oocyte extract system. Polyamide treatment did not cause activation of the ATR checkpoint or preclude binding of pre-RC factors. However, polyamide treatment did disrupt sperm chromatin decondensation, thus preventing initiation of DNA replication. Inhibition this early in the cell cycle in this model system limits its use in understanding how polyamides might inhibit active replication; however, inhibition of decondensation is an interesting result and may provide new insight into the mechanistic effects of polyamides.

Inhibition of chromatin decondensation in frog egg extracts has been shown to occur in response to many DNA-binding small molecules, including ethidium bromide, doxorubicin, and echinomycin (7,8). Sperm chromatin in extracts treated with ethidium bromide failed to fully decondense even after 90 minutes, though it is noticeably expanded in size compared to its initial size (8). This study concluded that modification to the DNA topology by ethidium led to inhibition of nuclear membrane formation and lamina assembly, which is necessary for the second phase of proper chromatin decondensation. Ethidium bromide treated extracts were also assessed for loading of pre-RC factors; the MCMs, PCNA, and RPA were all loaded despite a lack of nuclear membrane formation. This result is similar to our observations of polyamide treatment, except for significant loading of RPA. However, Krasinska et al. used GFP-fused proteins to assess DNA loading as opposed to chromatin fractionation. Inhibition of

decondensation by ethidium bromide was also accompanied with inhibition of nucleotide incorporation but no activation of the DNA checkpoints, consistent with the effects observed by polyamide treatment. Given that a variety of DNA-binding small molecules similarly inhibit DNA decondensation, there must be a common mechanism related to distortion of the structure of DNA causing this effect.

Py-Im polyamides are unique compared to other DNA-binding small molecules because they can be designed in a modular fashion in order to target specific sequences of DNA. However, this study shows that they still share many of the same effects as other DNA-binding molecules despite each having different binding modes with the DNA; ethidium bromide is an intercalator, doxorubicin has a mixture of intercalation and minor groove interactions, echinomycin is a bis-intercalator with minor groove interactions, and polyamides bind in the minor groove. Interestingly, The parent molecule of Py-Im polyamides, the minor groove binder distamycin A, has been shown to alter the structure of soluble chromatin resulting in compaction of chromatin (9). Majumder *et al*. show by electron microscopy that individual nucleosomes in di- and tri-nucleosomes are pulled close together under treatment with distamycin. This result may explain how polyamide binding inhibits decondensation of sperm chromatin in egg extracts. In a separate study, distamycin has also been shown to induce remodeling of a chromatosome by eviction of the linker histone H1 (10). This effect is also suggested to be due to torsional stress induced by distamycin binding to DNA and might provide insight into the phenomenon caused by polyamides in egg extracts. Similar investigations into the effects of Py-Im polyamides on chromatin structure and DNA topology would likely prove useful in

explaining how chromatin decondensation is inhibited and perhaps even how DNA replication and transcription are inhibited generally.

While the CSF arrested extracts were not suitable as a model to test whether polyamides can inhibit the progression of replication forks, *Xenopus* oocyte extracts can be prepared in a different manner that allows for the replication of exogenous plasmids without the need for nuclear formation. In this system, egg extracts are prepared by high speed ultracentrifugation and mixed with a concentrated nucleoplasmic extract (NPE) (11). The ability to use exogenous plasmids for the DNA template bypasses the DNA decondensation step and also allows for the DNA sequence to be controlled such that any number of polyamide match sites can be incorporated in order to test for sequence dependence.

Another method to bypass the chromatin decondensation and nuclear membrane formation steps would be to use a hybrid *Xenopus*-mammalian cell system. This method uses the nuclei from G1 synchronized mammalian cells, which have not yet initiated DNA replication, and incubates these with frog egg extracts. May *et al*. use this technique to demonstrate that echinomycin inhibits the DNA replication of human chromosomes after failing to proceed past the decondensation step using frog sperm chromatin (7). Cell culture studies in DU145 cells or other cell lines can also provide more insight into polyamide effects on the cell cycle by employing synchronization. Through a variety of means (Figure 3.6), cells can be synchronized at each phase of the cell cycle and then dosed with polyamides to determine what effects are caused relative to

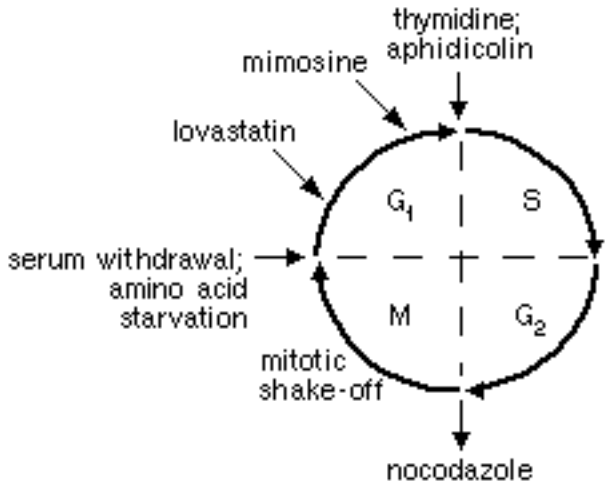

Figure 3.6 Methods of synchronizing mammalian cells at different stages of the cell cycle (12).

each phase (12). Future studies employing these methods will be invaluable for understanding how Py-Im polyamides induce replication stress and potentially cell death or reduced tumor growth in animal models.

3.4 Materials and Methods

Chemicals and reagents.

Hairpin Py–Im polyamides were synthesized on solid phase Kaiser oxime resin using previously published protocols (13). Aphidicolin, actinomycin D, and distamycin A were purchased from Sigma-Aldrich, as were all other reagents unless otherwise noted. Anti-Chk1 antibody was purchased from Santa Cruz Biotech. Anti-Chk1 pS345 (cross-reacts with Xchk1 pS344) was purchased from Cell Signaling Technology.

Preparation of X. laevis oocyte extracts.

Female frogs were primed with pregnant mare serum gonadotropin (PMSG) 3 days prior to induction with human chorionic gonadotropin (HCG, Sigma) as described in

(14). Cytostatic factor-arrested low speed supernatant extracts from healthy unactivated *Xenopus* eggs and demembranated sperm chromatin were prepared as also described in (14). Interphase egg extracts were prepared by addition of $CaCl_2$ (0.4 mM). Egg extracts used for the preparation of nuclear and chromatin fractions were also incubated with 100 µg/mL cycloheximide to inhibit cyclin synthesis and subsequent entries into mitosis. The *Xenopus* Chk1 protein was radiolabeled *in vitro* with the TnT system (Promega, Madison, WI) in the presence of TRAN35S-LABEL (MP Biomedicals, Santa Ana, CA).

Measuring DNA replication by the incorporation of [α-^{32}P]-dATP.

The replication was run similarly to (15). Freshly prepared extract was mixed with 3,000 nuclei/µL and polyamide dissolved in DMSO at the indicated dose. The final DMSO concentration was 1%. 0.02 µCi of radiolabelled [α-^{32}P]-dATP was also added to each reaction for continuous labeling of DNA replication. The cell cycle was activated with the addition of 0.4 mM $CaCl_2$ and allowed to incubate for 100 min. When the reaction was complete, 10 µL was removed and mixed with 10 µL sample buffer (80 mM Tris HCl, pH 8.0, 8 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 5% sodium dodecyl sulfate, and 0.2% bromophenol blue) to quench the reaction. Samples were then treated with 1 mg proteinase K per mL for 1 h at 37ºC prior to gel electrophoresis on a 1% agarose gel. The gel was then dried and exposed on a storage phosphor screen for 1 h, followed by scanning on a Molecular Dynamics Storm 840.

Assessment of checkpoint activation and replication complex formation by immunoblots.

Fractionation of egg extracts into total nuclear and chromatin portions was performed as in (16). Nuclear and chromatin fractions from egg extracts were boiled in Laemmli buffer, and total protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4–15% gradient polyacrylamide gels (Bio-Rad). After transfer to the Immobilon-LF PVDF membrane (Millipore) and blocking with Odyssey Blocking Buffer (Li-Cor), primary antibodies were incubated overnight at 4ºC. After washing the membrance three times in PBST, 1:3000 donkey anti-rabbit or donkey anti-mouse 800CW IR dye-conjugated secondary antibody (Li-Cor) was added for 1 h at room temperature and the bands were visualized on an Odyssey infrared imager (Li-Cor).

3.5 Acknowledgments

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3.6 References

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