SUPPLEMENTARY DATA FOR

Replication Stress by Py-Im Polyamides Induces a Non-canonical ATR-dependent Checkpoint Response

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Supplementary Table 1. Summary of cytotoxicity IC$_{50}$ values of polyamides 1 and 2 in AR-overexpressing (+++, LNAR), AR-expressing (+, LNCaP), and AR-negative (-, DU145) cancer cell lines. Cells were treated continuously with polyamides for 72 or 96 h before fixation and staining. Values represent the mean ± S.D. of three replicates.

| Cell line | AR   | 1  | 2  |
|-----------|------|----|----|
|           | 72h  | 96h| 72h| 96h|
| LNAR      | +++  | 40±10 | 36±14 | 3±1 | 1.5±0.2 |
| LNCaP     | +    | 18±4 | 7±3 | 1.8±0.9 | 0.6±0.2 |
| DU145     | -    | 14±4 | 8±4 | 1.5±0.5 | 0.76±0.06 |
Supplementary Figure 1. Polyamides induce apoptosis in DU145 cells. (A) Cell viability assay. Cells were treated in quadruplicate with polyamide 1 (top) or polyamide 2 (bottom) for range of concentrations (µM) for up to 96 h and then assayed for bioreductive capacity with WST-1 reagent. The data are normalized to the untreated condition. (B) Caspase 3/7 activity assay. Cells were treated in triplicate with polyamide 1 (top) or polyamide 2 (bottom) for the indicated time and then homogenized in guanidinium lysis buffer containing a pro-luminescent Caspase 3/7 substrate. The data are normalized to the untreated condition. (C) ELISA for cleaved PARP formation. Cells were treated with polyamides for 72 h. before assaying the lysates by sandwich ELISA using an HRP-conjugated secondary antibody and a chromogenic substrate. The data are presented as the background-corrected absorbance values at 450 nm. Error bars represent the mean ± S.D. of experiments conducted in triplicate or quadruplicate.
**Supplementary Figure 2.** Effects of small molecule PI3-kinase inhibitors on polyamide-induced S-phase accumulation. Cell cycle distribution of DU145 cells after 36 h treatment with DMSO, 10 µM polyamide 1, or 1 µM polyamide 2 in the presence of 2 mM caffeine, 10 µM KU55933 (KU, ATM inhibitor), or 10 µM NU6027 (NU, ATR inhibitor) as measured by single-color flow cytometric evaluation of propidium iodide stained cells. When both KU and NU were added together with DMSO or polyamide, only 4 µM of each inhibitor was used to reduce toxicity.
Supplementary Figure 3. High concentration polyamide treatment does not inhibit aphidicolin-induced Chk1 phosphorylation. (A) Immunoblot of Chk1pS345 after the treatment with polyamides followed by aphidicolin. DU145 cells were treated with DMSO, 10 µM polyamide 1, or 1 µM polyamide 2 followed by the addition of 10 µg/mL aphidicolin (Aph) after 24 h. Cells were harvested after 36 h total incubation. (B) Immunoblot of Chk1pS345 after simultaneous treatment of DMSO or 3 µM polyamide 2 plus 10 µg/mL Aph for 12 or 24 h. (C) Immunoblot of S-phase checkpoint and DNA damage response proteins, Chk1pS345, RPA2pS4/8, Chk2pT68, and γ-H2AX in DU145 cells after 18 h treatment with DMSO, 30 µM etoposide, polyamide 1, or polyamide 2 at the indicated concentrations.
Supplementary Figure 4. Effects of ATM- and ATR-specific small molecule inhibitors on polyamide-induced MCM2 S108 phosphorylation and FANCD2 monoubiquitination. (A) MCM2 S108 phosphorylation levels were measured in DU145 cells treated with 4 mM HU for 2 h, and DMSO, 10 µM polyamide 1 or 1 µM polyamide 2 for 36 h in addition to 10 µM KU55933 (KU, ATM inhibitor), 10 µM NU6027 (NU, ATR inhibitor), or both KU and NU. Only 4 µM KU and 4 µM NU were used when both inhibitors were added together to reduce toxicity. (B) FANCD2-Ub levels were measured in selective kinase inhibitor-containing lysates. Monoubiquitination was estimated by normalizing the band intensity of the large molecular weight monoubiquitinated FANCD2 band (FANCD2-L) to the low molecular weight non-ubiquitinated FANCD2 band (FANCD2-S).
Supplementary Figure 5. FANCD2 increases cell survival after exposure to polyamide 1. PD20 cells complemented with empty vector (PD20-EV) or FANCD2 (PD20-FANCD2) were treated with the indicated concentrations of polyamide 1 for 36h and assayed for survival after 14 days. Error bars indicate mean ± SEM for n=3 independent experiments. Unpaired T-tests were performed at *P<0.05.
Supplementary Figure 6. Py-Im Polyamides stabilize duplex DNA regardless of match site position in the duplex. DMSO or 4 µM polyamide was incubated with 2 µM 14 bp duplex DNA containing only a single 5’-WGWWCW-3’ binding site positioned either 4 bps (A), 2 bps (B), or 1 bp (C) from the edge and a melting curve was measured using DNA hyperchromicity (59). The average melting temperature and standard deviation were calculated from four replicates.

|   | A                  | B                  | C                  |
|---|--------------------|--------------------|--------------------|
|   | 5’-TCGC AGAACA GCGA-3’ | 5’-GT AGAACA GCGACC-3’ | 5’-C AGAACA GCGTCG-3’ |
|   | 3’-AGGG TCTTGT CGCT-5’ | 3’-CA TCTTGT CGCTGG-5’ | 3’-G TCTTGT CGTCAGC-5’ |
|   |                    |                    |                    |
| $T_m$ (°C) | 61.7 (±0.4) °C | 61.4 (±0.3) °C | 61.3 (±0.5) °C |
| $\Delta T_m$ (°C) | 14.9              | 13.3              | 12.1              |
|   | 76.7 (±0.1)          | 74.8 (±0.1)          | 73.5 (±0.4)          |
|   | 76.2 (±0.2)          | 74.4 (±0.2)          | 74.2 (±0.3)          |
Supplementary Figure 7. Polyamide 1 inhibits helicase activity of *S. cerevisiae* Dna2 nuclease dead but helicase active mutant (yDna2-K677R) but not its ATPase activity. Inhibition of yDna2-K677R by polyamide 1 was tested using a forked DNA duplex containing either one match-binding site (A) or no match-binding site (B). $^{32}$P is represented in the cartoon of the substrate by the red asterisk. Polyamide 1 was added in increasing concentrations (lanes 4-14): 100 pM, 300 pM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 µM, 3 µM. (C) Graphical representation of yDna2-K677R inhibition curves. (D) 3 µM polyamide 1 was incubated with the single-stranded mismatch DNA oligomer and yDna2-K677R to assess whether polyamide 1 can inhibit yDna2-K677R ATPase activity.
Supplementary Figure 8. Dose-dependent increase in hydroxyurea (HU)-induced Chk1 S345 phosphorylation. Chk1 S345 phosphorylation was measured in DU145 cells treated with increasing doses of HU for 2 h. Chk1 S345 is maximally phosphorylated at 1 mM or higher HU.
REFERENCES

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