Modulation of topoisomerase IIα expression and chemosensitivity through targeted inhibition of NF-Y:DNA binding by a diamino *p*-anisyl-benzimidazole(Hx) polyamide

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Abstract

Background:

Sequence specific polyamide HxIP **1**, targeted to the inverted CCAAT Box 2 (ICB2) on the *topoisomerase IIα* (*topo IIα*) promoter can inhibit NF-Y binding, re-induce gene expression and increase sensitivity to etoposide. To enhance biological activity, diamino-containing derivatives (HxI*P **2** and HxIP* **3**) were synthesised incorporating an alkyl amino group at the N1-heterocyclic position of the imidazole/pyrrole.

Methods:

DNase I footprinting was used to evaluate DNA binding of the diamino Hx-polyamides, and their ability to disrupt the NF-Y:ICB2 interaction assessed using EMSAs. Topo IIα mRNA (RT-PCR) and protein (Immunoblotting) levels were measured following 18h polyamide treatment of confluent A549 cells. γH2AX was used as a marker for etoposide-induced DNA damage after pre-treatment with HxIP* **3** and cell viability was measured using Cell-Titer Glo®.

Results:

Introduction of the N1-alkyl amino group reduced selectivity for the target sequence 5'-TACGAT-3' on the *topo IIα* promoter, but increased DNA binding affinity. Confocal microscopy revealed both fluorescent diamino polyamides localised in the nucleus, yet HxI*P **2** was unable to disrupt the NF-Y:ICB2 interaction and showed no effect against the downregulation of topo IIα. In contrast, inhibition of NF-Y binding by HxIP* **3** stimulated dose-dependent (0.1-2 μM) re-induction of topo IIα and potentiated cytotoxicity of topo II poisons by enhancing DNA damage.

Conclusions:

Polyamide functionalisation at the N1-position offers a design strategy to improve drug-like properties. Dicationic HxIP* **3** increased topo IIα expression and chemosensitivity to topo II-targeting agents.

General Significance:

Pharmacological modulation of topo IIα expression has the potential to enhance cellular sensitivity to clinically-used anticancer therapeutics.

Keywords

DNA-binding polyamides

Sequence selectivity

Transcription factor-DNA interactions

Gene modulation

NF-Y

Topoisomerase IIα (Topo IIα)

Chemosensitisation

1. Introduction

Gene expression is precisely regulated by the binding of the transcription machinery to specific DNA sequences. Dysregulation of transcriptional activity leading to aberrant gene expression is a fundamental driver of a diverse array of human diseases. Sequence selective (P) Pyrrole - (I) Imidazole polyamides are able to modulate gene expression through binding non-covalently to specific DNA sequences and disrupting the DNA interactions of transcription factors. These reversible DNA minor groove binders can arrange in a stacked, antiparallel 2:1 (ligand:DNA) orientation and afford programmable sequence recognition, governed by the side-by-side heterocyclic ring pairing rules [1], [2], [3], [4]. A P/P pairing degenerately targets A•T or T•A, whereas P/I recognises C•G and I/P preferentially binds to G•C. The reported anti-cancer biological activity of these cell permeable small molecules in both cellular and *in vivo* studies has highlighted the potential of therapeutic strategies that directly target transcription factor-DNA interfaces known to be implicated in certain malignant phenotypes [5], [6], [7], [8].

Nuclear Factor Y (NF-Y) is a heterotrimeric CCAAT-binding transcription factor involved in cell differentiation, proliferation and implicated in cancer progression [9], [10], [11], [12], [13]. NF-Y has been shown to bind to the promoter of the essential DNA processing enzyme, topoisomerase IIα (topo IIα) and regulates its transcription through interactions with the inverted CCAAT box (ICB) sequences located within the promoter [14], [15], [16]. Topo IIα plays a critical role in DNA metabolism, maintaining genomic stability [17], and is the target of clinically-used chemotherapeutic agents etoposide and doxorubicin [18], with low levels of topo IIα conferring cellular resistance to these anticancer agents [19], [20], [21], [22]. NF-Y acts as both an activator and repressor of *topo IIα* transcription with increased association of NF-Y to the promoter exerting a negative effect at confluence [15], [16]. The ICB2 has been identified as the crucial DNA regulatory element and its interaction with NF-Y (Figure 1) mediates the confluence-induced downregulation of topo IIα and reduced chemosensitivity to topo II targeting therapeutics.

Chemical approaches that re-induce topo IIα expression have the potential to increase cellular sensitivity to topo II poisons and to this end, our group has used various DNA binding small molecules and polyamides to inhibit the repressive activity of NF-Y on the *topo IIα* promoter

[16], [23], [24], [25], [26], [27]. Most recently, we reported the synthesis and biological activity of a novel polyamide incorporating the *p*-anisylbenzimidazole (Hx) DNA recognition element [28], [29]. Designed to enhance polyamide-DNA binding and cellular uptake, the Hx moiety exhibits intrinsic fluorescence upon binding DNA enabling the direct visualisation of polyamide nuclear localisation. Hx-polyamide HxIP (**1,** Figure 2A) designed to target the 5'-flanking sequence 5'-TACGAT-3' of the ICB2 (Figure 1), binds to DNA with high affinity and sequence selectivity, and disrupts the NF-Y:ICB2 interaction resulting in the upregulation of topo IIα expression at confluence. HxIP pre-treatment enhanced etoposide-induced DNA damage, providing further evidence that sequence specific polyamides can re-sensitise confluence-arrested cancer cells to topo IIα poisons [28].

The development of HxIP provides a new framework for the design of fluorescent sequence selective DNA binding molecules, distinct in configuration from the prototypical hairpin polyamide, yet capable of efficient nuclear localisation and *in vitro* gene regulation. In parallel, we have continued to explore an alternative strategy for further optimisation of polyamide physicochemical and DNA binding properties, through the introduction of an additional alkyl amino group at the N1 position of the heterocyclic rings [30], [31], [32], [33]. DNA binding studies by our groups revealed the small diamino polyamide containing an orthogonal positioned propyl amino group, f-IP*I (*denotes modified heterocycle) to have greater binding affinity than its monoamino counterpart and analogous sequence selectivity. Importantly, the inclusion of an extra amino group, which is cationic at physiological pH, also increases water solubility and may afford greater polyamide nuclear uptake. We aim to exploit this potential combination of improved DNA binding and solubility properties to engineer a potentially more potent generation of dicationic polyamides.

This study presents the DNA binding and biological activities of the diamino Hx-polyamides HxI*P **2** and HxIP* **3**, which incorporate the N1-alkyl amino group modification (Figure 2A). Polyamides **2** and **3** are functionalised derivatives of HxIP **1** and like their monoamino predecessor are designed to target the ICB2 5'-flanking sequence 5'-TACGAT-3' on the *topo IIα* promoter (Fig 2B) and disrupt NF-Y binding, inducing topo IIα expression at confluence.

Comparison with the monoamino HxIP **1** will reveal the effect the inclusion of an N1-alkyl amino group in the Hx-framework has on DNA binding and the feasibility of using functionalization of the N1 position to enhance polyamide cellular uptake and biological activity. Additionally, we shall assess the potential chemosensitising effects of the diamino generation of polyamide inhibitors of NF-Y:DNA binding and the effectiveness of pharmacological modulation of topo IIα expression as a strategy for overcoming the drug resistance exhibited by confluence-arrested cells.

2. Methods and Materials

2.1 Synthesis of polyamides

Details of the synthesis and characterisation of diamino Hx-polyamides **2** and **3** are provided in the supplemental materials and methods (Scheme S1).

2.2 Thermal denaturation studies

Thermal denaturation (ΔT_M) studies were performed using a Cary Bio 100 spectrophotometer UV-Vis instrument (Palo Alto, CA) as previously described by Chavda et al [29]. DNA oligomers were purchased from Operon and the sequences are provided in the supplemental materials. Experiments for diamino Hx-polyamides **2** and **3** were performed at a concentration of 3 μM ligand and 1 μM DNA. All melts were performed in 10-mm path length quartz cells. T_M values were determined as the maximum of the first derivative, and ΔT_M values are the difference between the melting temperatures of ligand bound DNA and free DNA.

2.3 Circular dichroism

Circular dichroism (CD) studies were carried out as previously reported [30], using an OLIS (Bogart, GA) DSM20 spectropolarimeter. Experiments were conducted at ambient temperature in a 1-mm path length quartz cell using phosphate a $PO_4$0 buffer (10 mM sodium phosphate, 1 mM EDTA, pH 6.2). Buffer and stock DNA were added to the cuvette to give a final DNA concentration of 9 μM. Each diamino Hx-polyamide (in 500 μL in ddH_2O) was titrated in increments of 1 molar equivalent into the relevant DNA (160 μL of 9 μM DNA). Each run was performed over 400–220 nm. The CD response at the λ_{max} of the induced peak was plotted against the mole ratio of ligand to DNA.

2.4 Surface plasmon resonance (SPR)

Surface plasmon resonance (SPR) measurements were performed with a four-channel BIAcore T100 optical biosensor system (Biacore, GE Healthcare Inc.). A streptavidin-coated sensor chip was prepared by a series injections of 1 M NaCl in 50 mM NaOH for periods of 60 s followed by washing of the chip surface with HBS buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% P20, pH 7.4). Biotin-5'-end labelled DNA hairpin oligomers dissolved to 25 nM concentrations in HBS buffer were immobilized to the flow cell surface via non-covalent capture using previously described methods [34], [35]. The sequences of the three hairpin oligomers are detailed in the supplemental materials. Three flow cells were used to immobilise the DNA, while a fourth was left blank as a control. Immobilization was achieved by manual injection of the DNAs at a flow rate of 1 µL/min until response units (RU) of 350-400 were reached. Diamino polyamide **3** was dissolved in 500 µL MeOH and 0.9 mol eq. of HCl was added to form a salt. Methanol was removed using N_2 at 25 °C leaving a yellow salt. The compound was then re-dissolved in ddH_2O to a concentration near 1 mM and spectroscopically determined by UV-Vis using the extinction coefficient (ε_{322nm} = 29,129 $L \cdot mol^{-1} \cdot cm^{-1}$) and stored at 4 °C until use (within 2 weeks). Compound solution concentrations of **3** were prepared in 10 mM cacodylic acid (CCL), 1 mM EDTA, and 200 mM NaCl buffer (pH 6.5) ranging 2 nM - 1 µM and were injected over the sensor chip at a flow rate of 100 µL/min for 180 s. Buffer was then flowed over the chip surface for 600 s to dissociate bound HxIP* **3** from DNA. Following each cycle, the surface of the sensor chip was regenerated with 1 M NaCl for 30 s and rinsed with three injections of experimental buffer to produce a stable baseline for the following cycles. The relative response units (RU) were plotted as a function of free compound concentration (C_f) and equilibrium binding constants (K_{eq}) were determined using a two-site cooperative binding model with the following equation:

$$r = \frac{RU}{RUmax} = (K_1 \cdot C_f + 2\,K_1 \cdot K_2 \cdot C_f^2)/(1 + K_1 \cdot C_f + K_1 \cdot K_2 \cdot C_f^2) \quad (1)$$

Here, K_1 and K_2 are macroscopic binding constants in units per concentration (M^{-1}). The maximum obtainable response (RU_{max}) in equation (1) was calculated from the product of immobilized DNA response units, HxIP* molecular weight, refractive index of HxIP*, and the inverse DNA molecular weight. The obtained maximum value was compared to predict RU_{max} of bound HxIP* to determine the stoichiometry. Equilibrium binding constants (K_{eq}) were determined using both global kinetics fits and/or steady-state binding models. Two equilibrium

binding constants, K_1 and K_2, were determined, multiplied by each other, and the square root of the resulting calculated value gave an averaged equilibrium binding constant (K_{eq}) to compare with previous results [29].

$$K_{eq} = (K_1 \cdot K_2)^{1/2} \quad (2)$$

2.5 DNase I footprinting

The 5' radiolabelled DNA substrate corresponding to the *topo IIα* promoter was prepared by PCR amplification and then isolated and purified as previously reported [16]. The DNase I footprinting reactions were performed as described by Kiakos et al [28] and resolved on a 10% denaturing polyacrylamide-urea gel (National Diagnostics) by electrophoresis for 3 h at 1650 V (55°C) in 1x TBE buffer. The gel was then transferred onto Whatman 3MM paper, dried and exposed overnight to Fuji medical X-ray film to visualise the radioactive signal.

2.6 Cell lines and culture conditions

NIH3T3 mouse fibroblast cells were obtained from CR-UK London Research Institute, and the A549 cell line was purchased from the European Collection of Cell Cultures. All cell lines were maintained in DMEM (Sigma-Aldrich) supplemented with 2mM L-glutamine (Sigma-Aldrich) and 10% fetal bovine serum (Gibco, Life Technologies).

2.7 Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay (EMSA) experiments were conducted as outlined by Kiakos et al [28] using nuclear protein extracts from NIH3T3 fibroblast cells and the ICB2-containing oligonucleotide; 5'-GGCAAGCTACG*ATTGG*TTCTTCTGGACG-3' (sense); 5'-CGTCCAGAAGAACCAATCGTAGCTTGCC-3' (antisense). Oligonucleotides containing a mutated ICB2 were used as specific competitors, with the wild-type ICB motif replaced by AAACC and GGTTT in sense and antisense oligonucleotides respectively. For supershift

experiments the nuclear extract and radiolabelled oligonucleotide were incubated with anti-NF-YA antibody (Abcam) for 1h.

2.8 Immunofluorescence staining and confocal microscopy

To assess polyamide nuclear uptake, A549 and NIH3T3 cells grown on 13-mm glass cover slips were treated with different concentrations of polyamide **2** and **3** for various incubation times. Cells were fixed (2% PFA), washed with PBS and then permeabilised to allow nuclear DNA staining with PI (2 μg/ml). Alternatively, MitoTracker® Red was used to stain the mitochondria, without fixation or permabilisation. The blue fluorescence of the Hx-polyamides was excited with a UV-laser (364 nm) and detected using the DAPI filter. PI/ MitoTracker® Red was excited with an argon-ion laser (488 nm) and detected at 642 nm. The fluorescence of the polyamide was detected and imaged by z-stack acquisition using the Zeiss 510 UV-VIS microscope and the LSM510 software. Immunofluorescence was used to assay for H2AX foci induction after polyamide-etoposide combination treatment. A549 cells were washed with PBS, fixed with 2% PFA and permeabilised with PBS containing 0.5% Triton X-100. Cells were subsequently blocked in PBS 5% BSA for 1 h and incubated with anti-phospho-Histone H2A.X mouse monoclonal antibody (1:100; Millipore) diluted in PBS 1% BSA for 1h. After three washes, cells were incubated with the goat anti-mouse secondary antibody Alexa Fluor 488 (1:100; Life Technologies) for another hour. Following a further three washes in PBS 0.1% Triton X-100, nuclei were stained using a PI solution (2 μg/ml; Sigma-Aldrich). γH2AX levels were then visualised using confocal microscopy and the number of foci per nuclei was quantified using the CellProfiler software [36], [37].

2.9 Quantitative real time PCR

RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) and cDNA was generated with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real time PCR was

carried out using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) and Taqman assay probes (Life Technologies) for the target gene, *topo IIα* (Hs01032137_m1) and the internal control, *GAPDH* (Hs03929097_g1). The relative quantification of topo IIα mRNA levels from untreated and polyamide treated confluent A549 cells was determined using the 2^(-ΔΔCt) method and normalised to the internal control.

2.10 Immunoblotting

Nuclear extracts from A549 cell lines were prepared using a Nuclear Extraction Kit (Active Motif) following the manufacturer's protocol. Protein concentration was quantified using the Bio-Rad DC protein assay. Nuclear proteins were separated on a NuPAGE 7% Tris-acetate gel (Life Technologies), transferred to PVDF membranes and blocked (5% w/v non-fat dry milk in 1x TBS, 0.1% Tween-20) for 1 h at ambient temperature. Topo IIα was identified by overnight incubation with topo IIα rabbit polyclonal antibody (1:5000, kindly provided by Dr I.D. Hickson, Weatherall Institute of Molecular Medicine, Oxford, UK) and subsequent incubation for 1 h with anti-rabbit secondary antibody. Chemiluminescent visualisation was performed using ECL reagent (Amersham) and exposure onto autoradiography film (Kodak-X-Omat). Protein levels of γH2AX were also assessed after polyamide-etoposide combination treatment. For the detection of phosphorylated histone H2AX (Ser139), proteins were separated using a NuPAGE 4-12% Bis-tris gel and probed using the anti-phospho-histone H2AX monoclonal antibody (1:500, Millipore). Lamin (Cell Signalling) was used as a loading control for the nuclear protein extracts.

2.11 Cell viability

The Cell Titer-Glo luminescent assay (Promega) was used as per manufacturer's instructions to assess viability of A549 cancer cells after combination treatment with polyamide **3** and etoposide (400 μM) or doxorubicin (75 μM).

2.12 Statistical analysis

For statistical analysis of significance, data were analysed using either the unpaired, two-tailed students t-test or one-way analysis of variance (ANOVA) as appropriate. Results were considered statistically significant at a p value < 0.05 and were calculated using GraphPad Prism 6.

3. Results

3.1 DNA binding affinity and sequence selectivity of diamino Hx-polyamides 2 and 3

The DNA binding properties of the diamino Hx-polyamides **2** and **3** were investigated to assess

the effect of introducing an alkyl amino group at the heterocyclic N1 position. Thermal denaturation analysis probed the binding affinity and selectivity of polyamides **2** and **3** by measuring their ability to stabilise duplex DNA. The Hx recognition element behaves similarly to two consecutive pyrrole units and as a result, polyamides **1**, **2** and **3** can degenerately bind sequences 5'-ATCGAT-3' and 5'-TACGAT-3', with the latter found on the 5'-flank of the ICB2. The ΔT_M data presented in Table 1 indicate that polyamides **2** (ΔT_M = 30°C) and **3** (ΔT_M = 32°C) bind to the target sequence (5'-ATCGAT-3') with greater affinity than their monoamino counterpart **1** (ΔT_M = 15°C), displaying a 2-fold increase in ΔT_M relative to HxIP **1** [29]. These results show that the additional positively charged alkyl amino group contributes favourably to DNA stabilisation. However, polyamides **2** and **3** also showed increased ΔT_M values for the non-cognate sequences 5'-ACGCGT-3' and 5'-AAATTT-3', suggesting an overall decrease in sequence selectivity relative to **1**, which has previously been shown to have little effect on the stabilisation of non-cognate sequences [29]. The increased stabilisation of the oligonucleotides by dicationic Hx-polyamides **2** and **3** demonstrated by thermal denaturation is consistent with previously published of comparable N1-derivaitized dicationic polyamides [32], [33].

Circular dichroism (CD) studies confirmed that both diamino Hx-polyamides **2** and **3** bind effectively to the 5'-ATCGAT-3' target sequence in the minor groove (Figure 3A), as shown by the emergence of a strong DNA-induced ligand band at ~330 nm. The observation of a clear isodichroic point at 305 nm suggests that **2** and **3** bind through a single mechanism, in a presumed side-by-side, antiparallel, stacked orientation. However, HxIP* **3** induced a strong CD band and clear isodichroic point when titrated to the non-cognate sequences, demonstrating that DNA sequence selectivity is reduced by the presence of the second alkyl amino group and corroborating the findings from the DNA denaturation experiments. HxI*P **2** also bound to the non-cognate sequences, but overlaid CD spectra showed that the CD bands were weaker and the isodichroic points less distinct than those observed for **3**, confirming the overall lower binding affinity of polyamide **2** and highlighting that the position of the N1 modification affects DNA binding affinity.

To further investigate the effect of the N1-alkyl amino group, the binding constants (K_{eq}) of the diamino Hx-polyamide **3** were determined using the surface plasmon resonance (SPR) biosensor assay. The sensorgrams and steady-state plots shown in Figure 3B/3C, revealed that HxIP* **3** binds to the consensus 5'-ATCGAT-3' site with high affinity ($K_{eq} \approx 3 \times 10^6$ M^{-1} or a K_D of approximately 0.3 μM). Interestingly, diamino Hx-polyamide **3** also binds to the non-cognate sequence 5'-ATGCAT-3' ($K_{eq} \approx 2 \times 10^6$ M^{-1}) almost as strongly as to the consensus sequence, but showed little affinity for 5'-ACGCGT-3' ($K_{eq} < 10^5$ M^{-1}). The favourable binding of Hx-polyamide 3 to the non-cognate sequence 5'-ATGCAT-3' could be due to strong electrostatic interactions between the protonated alkyl amino groups and the negatively charged phosphodiester backbone of DNA, which could compromise the weaker forces, such as hydrogen bonding and hydrophobic interactions, that govern sequence selectivity. Alternatively, we cannot rule out the possibility that a stacked, anti-parallel dimer of Hx-polyamide 3 could interact with 5'-ATCGAT-3' in a reversed alignment of 3'-to-5', rather than the typical 5'-to-3' manner.

3.2 Binding of diamino HxI*P and HxIP* on the *topo IIα* promoter

DNase I footprinting studies on the biologically relevant *topo IIα* promoter (Figure 4) reveal that HxI*P **2** binds to the 5'-TACGAT-3' target sequence of the 5'-flank of the ICB2 with weaker affinity (3 μM) than the monoamino HxIP **1** (1 μM, [28]) despite showing greater affinity in the biophysical studies. HxIP* **3** displayed a 2-fold enhancement of binding affinity relative to polyamide **1**, generating a footprint evident at 0.5 μM, which is in strong agreement with the SPR results. Incorporating an additional alkyl amino group had a detrimental effect on the sequence selectivity, with both polyamides **2** and **3** showing off-target binding at a sequence (5'-TTGGTT-3') overlapping the ICB3.

3.3 Disruption of the NF-Y:ICB2 interaction by diamino Hx-polyamides

The diamino polyamides inhibit the NF-Y binding to the ICB2 in a cell-free system with contrasting effectiveness as shown by electrophoretic mobility shift assay (EMSA) experiments (Figure 5). A radiolabelled oligonucleotide containing the ICB2 and target flanking sequence 5'-TACGAT-3' was incubated with nuclear protein extract from NIH3T3 cells after pre-treatment with HxI*P **2** or HxIP* **3**. Supershift studies using an antibody specific for NF-YA confirmed the presence of NF-Y within the ICB2-bound protein complex (Figure 5C). HxI*P **2** only affected NF-Y binding at higher concentrations of 10-20 μM, whereas HxIP* **3** displayed a dose-dependent inhibition of the NF-Y:ICB2 interaction, evident at doses ≥3 μM. In addition, diamino HxIP* **3** was able to displace NF-Y already bound to the ICB2 (Figure 5B), when the radiolabelled oligonucleotide was incubated with the nuclear extract prior to the addition of the polyamide. The comparative EMSA study of polyamides **2** and **3** emphasises the importance of optimising the DNA binding affinity and sequence selectivity to deliver polyamide-directed interference of specific protein-DNA interactions.

3.4 Nuclear uptake of diamino Hx-polyamides

Emission studies confirmed that the diamino-containing HxI*P **2** and HxIP* **3** retain the characteristic fluorescent output exhibited by Hx-polyamides upon binding DNA and UV excitation (322 nm), with emission bands detected at 370 and 375 nm for compounds **2** and **3**, respectively (data not shown). Previously reported cellular uptake studies of HxIP **1** utilised the intrinsic fluorescence of the Hx fluorophore to visualise the rapid polyamide localisation in the nucleus [28]. Confocal microscopy and flow cytometry experiments were also used in this study to assess the nuclear uptake of polyamides **2** and **3**. A549 and NIH3T3 cells were treated with increasing concentrations of polyamides for 24 h and representative confocal microscopy images are shown in Figure 6. Nuclear uptake of **2** and **3** was confirmed by co-localisation of the polyamide fluorescence signal (blue) and propidium iodide (PI) signal (red). HxI*P **2** and HxIP* **3** nuclear staining increased in a dose-dependent manner, most prominent after exposure to 20 μM of each polyamide. Flow cytometry analysis (Figure S2) confirmed the confocal microscopy findings, with a 24 h exposure to each diamino polyamide resulting in a

concentration-dependent increase in the median fluorescence intensity of the distribution of the treated cell populations when compared to the DMSO-treated controls. A 41-fold and 26-fold increase was observed after treatment with HxI*P **2** and HxIP* **3**, respectively. It is not clear whether this difference in fluorescent output is due to the enhanced nuclear uptake of **2** or because of lower sequence selectivity, leading to promiscuous nuclear DNA binding and therefore greater fluorescence signal.

To examine polyamide uptake in live, unfixed NIH3T3 cells, the MitoTracker® Red dye, which stains the mitochondria, was used to define the cytoplasm without cell fixation or permeabilisation. The preferential accumulation of the polyamides in the nucleus was observed in NIH3T3 cells, with no overlap between the diamino polyamides and the MitoTracker fluorescence signals (Figure S3A). A time course experiment revealed the rapid nuclear uptake and sustained localisation of both diamino analogues following exposure to 20 μM, with blue fluorescence signal visible after just 1 h and still evident after 48 h (Figure S3B & C). These results highlight the advantage of incorporating the fluorogenic Hx moiety to monitor cellular uptake.

3.5 Biological activity of diamino Hx-polyamides in A549 cells

To explore whether the introduction of a second alkyl amino group enhanced polyamide biological activity, topo IIα expression in confluent A549 cancer cells was evaluated by measuring topo IIα mRNA and protein levels after polyamide treatment (Figure 7). HxIP* **3** induced a dose-dependent increase in topo IIα mRNA levels as shown by Quantitative RT-PCR, with expression upregulated by 2.1-fold after 18 h treatment with 2 μM polyamide. In contrast, HxI*P **2** had no effect on topo IIα mRNA expression at the same concentration. Immunoblot analysis confirmed the differing biological activities of the diamino HxIP analogues. Treatment with HxIP* **3** mediated a concentration-dependent (0.1-2 μM) enhancement of topo IIα protein levels relative to the untreated cells, with a stimulatory effect exerted at 0.1 μM, whereas, HxI*P **2** had no effect on the nuclear topo IIα content (Figure 7B). With both diamino

polyamides confirmed to localise in the nucleus via confocal microscopy, these findings indicate that the strikingly different *in vitro* biological activities displayed by **2** and **3** are dictated by the position of the additional alkyl amino group and its effect on DNA binding. Importantly, topo IIα expression in A549 cells is upregulated by the dicationic polyamide **3** at a lower concentration than reported for the monocationic polyamide **1** [28]. The enhanced polyamide efficacy may be a consequence of increased DNA binding affinity and water solubility arising from the inclusion of a second positively charged amino group.

3.6 HxIP*- mediated chemosensitisation to topo II poisons

The NF-Y-induced transcriptional downregulation of topo IIα at confluence results in cellular resistance to anti-cancer therapeutics targeting the activity of the enzyme. Previously, we reported that the re-induction of topo IIα expression after treatment with HxIP **1** re-sensitised confluent A549 cells to the DNA damaging effects of etoposide, as shown by the increased levels of DNA damage marker, γH2AX [28]. Here, we show the effects of combining topo II poisons with the more biologically active polyamide, HxIP* **3** (Figure 8). Following pre-treatment of confluent A549 cells with increasing concentrations of HxIP* for 24 h, cells were exposed to 50 μM etoposide for 2 h and allowed to recover in drug-free medium for a further 24 h before visualization of γH2AX. Representative immunofluorescence confocal microscopy images shown in Figure 8A revealed that HxIP*-etoposide combination treatment regimes initiated a polyamide dose-dependent increase in the number of γH2AX foci, relative to the etoposide treatment alone. The mean number of foci per nuclei was quantified (Figure 8B) using CellProfiler Software [36], [37]. Compared to etoposide alone (~0.46 γH2AX foci), combination treatments with 1 μM (~ 1.6 γH2AX foci) and 2 μM HxIP* (~ 2.5 γH2AX foci) induced statistically significant 3.5-fold and 5.4-fold increases in γH2AX foci, respectively (Figure 8B). HxIP* treatment resulted in negligible levels of γH2AX as it does not induce DNA damage due to the non-covalent binding nature of polyamides. Immunoblot analysis (Figure 8C) corroborated these findings and after pre-treatment with HxIP* there was a concentration-dependent increase in the phosphorylation of H2AX relative to etoposide alone, with the most significant

increase observed after pre-treatment with 5 μM HxIP*. A sustained, dose-dependent upregulation of nuclear topo IIα expression by HxIP* was observed in parallel and correlated with the enhanced levels of γH2AX.

HxIP* can mediate the chemosensitisation of confluent A549 cells to the cytotoxic effects of etoposide and doxorubicin. Cell viability was assessed following single agent HxIP* or etoposide treatment, and after HxIP*-etoposide combinations, where 6 h etoposide exposure followed 24 h pre-treatment with HxIP*. Combination of etoposide with increasing concentrations of HxIP* caused a synergistic dose-dependent decrease in cell viability relative to the untreated control. The combination of etoposide (400 μM) with 5 μM HxIP* reduced cell viability by an additional 50% compared to cells treated with etoposide alone (Figure 8D). HxIP* alone had little effect on viability and displayed no cytotoxic effects below 100 μM in exponential or confluent A549 cells following 24 h treatment (Figure S4A). Where as, etoposide induced-cytotoxicity following 6 h exposure was attenuated in confluent-arrested cells (Figure S4B), further demonstrating the confluence-mediated resistance to topo II poisons. Finally, polyamide induced cellular sensitisation was also observed in combination with doxorubicin (Figure 8E). Pre-treatment with 5 μM HxIP* enhanced doxorubicin induced-cytotoxicity causing an additional 22% decrease in viability relative to cells treated with doxorubicin (75 μM) alone. These results confirm the potential application of NF-Y modulating polyamides as chemosensitising agents to increase the cytotoxic potency of topo II poisons and reverse the resistance of confluence-arrested cancer cells.

4. Discussion

DNA binding polyamides with programmed sequence recognition are able to chemically control transcription and their gene regulatory activities have been confirmed in various biological

contexts, targeting a range of transcription factors such as nuclear hormone receptors [6], [8], hypoxia-inducible factor 1 (HIF-1) [38], nuclear factor κβ (NF-κβ) [7] and c-Myc [39]. Nevertheless, design strategies that further enhance polyamide activity are still required in order to realise the therapeutic potential of this class of small molecules. Recent advances within the field have centred on improving the often-modest nuclear uptake properties, which is essential for polyamide activity as gene control agents [40], [41], [42], [43]. We reported that the incorporation of the *p*-anisylbenzimidazole (Hx) DNA recognition element into a simple triamide structure increases nuclear uptake and the subsequent biological activity of the polyamide HxIP **1**, targeting the repressive DNA binding of transcription factor NF-Y to the ICB2 of the *topo IIα* promoter [28]. Here, we present the continued evolution of NF-Y targeting polyamides, and explore the modification of the Hx-polyamide framework through the introduction of an alkyl amino group at the N1 position of either the imidazole (HxI*P **2**) or pyrrole (HxIP* **3**).

This approach to polyamide functionalization was driven by the higher binding affinity and more effective inhibition of transcription factor-DNA interactions by polyamides integrating pyrrole N1-alkyl spermine/spermidine groups [44], [45]. The results of ΔT_M and CD studies confirm that the presence of the N1-alkyl amino group increases binding affinity of the diamino Hx-polyamides **2** and **3** to the cognate sequence, because of electrostatic interactions between the additional positively charged amino group and the negatively charged phosphodiester groups of the DNA backbone. However, the position of the N1 modification also affected the binding affinity of isomers **2** and **3**. The inclusion of the propyl amino group at the C-terminal heterocycle of the HxIP design (HxIP* **3**) caused a greater enhancement of binding affinity than derivatisation of the central heterocycle (HxI*P **2**). The difference in affinities between the two diamino analogues became more apparent following footprinting studies to assess their binding to the biologically relevant 5'-TACGAT-3' sequence on the *topo IIα* promoter. Diamino HxIP* **3** displayed greater binding affinity (0.5 μM) than the monoamino HxIP **1** (1 μM) [28], whereas HxI*P **2** binds to the target sequence with reduced affinity (3 μM). The weaker binding affinity of polyamide **2** relative to **3** may be due to the closer proximity of the cationic N1-alkyl amino groups of the stacked HxI*P **2** dimers when bound in a 2:1 configuration. Electrostatic

repulsions between the positively charged groups and steric effects between the alkyl chains could cause non-optimal polyamide-DNA binding orientations and compromise DNA binding affinity. N1 modification to C-terminal pyrrole on the other hand, limits the electrostatic and steric clash as the propyl amino groups of the stacked HxIP* **3** dimers are positioned at the maximum possible distance apart.

The importance of the position of the orthogonal propyl amino group in the polyamide design was previously revealed when the binding affinities of 5'-ACGCGT-3' targeting diamino triamides were shown to be dependent upon the position of the modified heterocycle, without significantly affecting DNA sequence selectivity [30], [31], [32], [33]. In contrast, the diamino Hx-polyamides **2** and **3** both display reduced DNA sequence selectivity relative to monoamino **1**, with the integration of the N1-alkyl amino group at the imidazole (HxI*P **2**) further compromising selectivity compared to attachment to the pyrrole ring (HxIP* **3**). This reduced selectivity is attributed to the increased positive electrostatic potential of the diamino polyamides, due to the presence of a second cationic moiety, resulting in greater attraction to the negative potential in the minor groove of AT rich sequences. These findings demonstrate how modifications can dramatically influence polyamide-DNA binding characteristics, and underlines the importance of functionalities which can enhance DNA binding affinity without affecting sequence selectivity to deliver optimised binding properties.

Engineered to derepress the NF-Y-mediated downregulation of topo IIα, the diamino Hx-polyamides displayed markedly different *in vitro* biological activities, seemingly dictated by the position of the N1-alkyl amino group and its effect on the polyamide's ability to disrupt the repressive NF-Y:ICB2 interaction. HxI*P **2** had no effect on topo IIα expression at mRNA or protein levels in A549 cancer cells, whereas HxIP* **3** induced upregulation in a dose-dependent manner. Previously, polyamides that displayed no biological effect were assumed to be incapable of penetrating into the nucleus [27]. Here, confocal microscopy and flow cytometry studies exploiting the inherent fluorescence of the diamino Hx-polyamides confirmed the rapid and sustained nuclear accumulation of both analogues. Therefore, the inability of HxI*P **2** to control topo IIα transcription can be attributed to the polyamide's attenuated DNA binding

properties and the resulting moderate inhibition of the NF-Y:DNA interaction. In contrast, diamino polyamide **3** not only exhibited a greater biological effect than **2**, but also stimulated upregulation of topo IIα at a lower concentration than the monoamino HxIP **1**[28]. EMSA studies revealed HxIP* **3** and HxIP **1** inhibited the NF-Y:ICB2 interaction with comparable efficiency despite footprinting studies showing diamino polyamide **3** to bind to the target sequence 5'-TACGAT-3' with higher affinity [28]. This suggests that the greater induction of topo IIα expression by HxIP* **3** is not solely a result of superior DNA binding. The presence of an additional cationic functionality also increases the aqueous solubility of diamino Hx-polyamides. Taken together, these results indicate that the improved biological effects of diamino HxIP* **3** derive from improved physicochemical properties including enhanced water solubility arising from the inclusion of a second positively charged alkyl amino group.

The introduction of a N1-alkyl amino group and its position influenced the DNA binding and biological activities of diamino Hx-polyamides **2** and **3**, and highlights why polyamide functionalisation has been widely explored as a strategy to advance their therapeutic potential (reviewed in [46]). Dervan and co-workers have reported on the effect of altering the composition of the C-terminal tail group of non-fluorescent polyamides [40], [41], [47]. The introduction of an isophthalic acid (IPA) group at the C-terminus preserved DNA binding affinity and selectivity, and increased potency in cell culture [41]. The attached IPA group enhanced nuclear localisation and enabled non-conjugated polyamides to replicate the efficient uptake of fluorophore-conjugated derivatives to deliver greater biological effects. An additional adjustment to the C-terminus group via the substitution of the amide linkage with an oxime linkage between the aliphatic linker and the aromatic tail group further enhanced the potency of polyamides targeting the androgen response element [42]. In a different approach, Sugiyama's group tested the introduction of a hydrophilic methoxypolyethylene glycol (PEG) 750 group as a strategy to address the poor aqueous solubility of their hairpin polyamide *seco*-CBI conjugates [48]. Conjugates modified by PEGylation at the hydroxyl group of the seco-CBI moiety showed moderately higher solubility and caused greater cytotoxic effects in A549 and DU145 cell lines due to improved cell permeability. Furthermore, work in Dervan's group showed that variation of the hairpin polyamide γ-aminobutyric acid turn (γ-turn) significantly

affected the biological efficacy, pharmacokinetics and toxicity [43], [49], [50]. The integration of an aryl group at the β-position of the γ-turn enhanced biological activity against nuclear receptor mediated transcription by two orders of magnitude [43]. However, further studies confirmed that modifications to the α- and β-positions of the γ-turn dramatically altered systemic toxicity of polyamides in mice [49], [50].

The inclusion of a second alkyl amino group did not change the inherently non-cytotoxic properties of the Hx-polyamides and no evidence of polyamide-induced DNA damage was detected after exposure to diamino polyamide **3**. However, when used in combination with etoposide and doxorubicin, HxIP* **3** stimulated the chemosensitisation of confluence-arrested cancer cells to the DNA damaging effects of these topo II targeting agents. Enhancing cellular sensitivity to the cytotoxic effects of topo II poisons through abrogation of the repressive NF-Y binding to the ICB2 was first demonstrated using *bis*-benzimidazole minor groove binder Hoechst 33342 [16]. Upregulation of topo IIα expression in confluent mouse fibroblast NIH3T3 cells significantly lowered the IC_{50} value of etoposide in combination treatments and inspired the development of sequence specific DNA interacting agents with greater selectivity for the critical ICB2 sequence. Hairpin polyamide JH-37 bound to the 5'-TTGGT-3' sequence overlapping the ICB2 and ICB3, and increased formation of DNA strand breaks when NIH3T3 cells were exposed to polyamide-etoposide treatments, resulting in a synergistic reduction in cell viability [25]. Recently, HxIP **1** was the first ICB2-targeting polyamide to sensitise confluent cancer cells to etoposide [28]. Here, diamino HxIP* **3**, our most potent NF-Y inhibiting polyamide to date, re-activated topo IIα expression and the subsequent increase of etoposide-generated DNA damage significantly reduced cell viability in combination treatments. The enhancement of etoposide and doxorubicin induced-cytotoxicity by diamino Hx-polyamide **3** reaffirmed the feasibility of direct modulation of transcription factor NF-Y activity as an approach to chemosensitisation within the context of cellular confluency.

5. Conclusion

Polyamide functionalisation via the N1 position of the pyrrole and imidazole rings in the Hx-framework presents a promising approach to improving the drug-like properties of these small molecules. The introduction of an additional cationic alkyl amino functionality enhances water solubility and facilitates the nuclear uptake of the diamino polyamides **2** and **3**. However, the N1-alkyl amino group had a detrimental effect on DNA sequence selectivity, and binding affinity for the target sequence 5'-TACGAT-3' on the *topo IIα* promoter was dependent upon the position of the modified heterocycle. The diamino polyamides displayed strikingly different *in vitro* biological activities. HxI*P **2** showed no effect against the downregulation of topo IIα at confluence, while HxIP* **3** stimulated a dose-dependent upregulation of topo IIα expression. This discrepancy is seemingly dictated by their contrasting abilities to disrupt the NF-Y:ICB2 interface. Overall, the correct positioning of a second alkyl amino group in the HxIP structure generated a more potent dicationic polyamide, benefiting from superior solubility relative to its monocationic counterpart. Targeted inhibition of NF-Y binding by HxIP* **3** and the subsequent re-induction of topo IIα levels potentiated the cytotoxicity of topo II poisons. These results further highlight the need for *in vivo* investigation of the efficacy of drug combination strategies that employ polyamides to overcome resistance to clinically-used anticancer therapeutics.

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Figure Legends

Figure 1: Schematic representation of the confluence-induced downregulation of topo IIα, mediated by the repressive binding of NF-Y to the ICB2. The ICB sequence ATTGG is highlighted in blue and the 5'-flanking sequence of the ICB2 is outlined with a dashed box.

Figure 2: Design of the diamino Hx-polyamides **2** and **3**. (**A**) Structures of the monoamino Hx-polyamide, HxIP **1** and the orthogonally positioned diamino-containing derivatives, HxI*P **2** and HxIP* **3**. The asterisk (*) denotes position of the N-alkyl amino group. (**B**) Schematic models of

polyamides **2** and **3** binding to the 5'-TACGAT-3' sequence on the 5'-flank of the ICB2 in an antiparallel 2:1 fashion as an overlapped stacked dimer.

Table 1: Thermal denaturation values and SPR equilibrium binding constants for polyamides HxIP **1**, HxI*P **2** and HxIP* **3**.

Figure 3: Biophysical comparison of the diamino Hx-polyamide DNA binding characteristics. (**A**) CD spectra for HxIP* **3** (top panel) and HxI*P **2** (bottom) binding with the target sequence 5'-ATCGAT-3' and non-consensus sequences 5'-AAATTT'-3 and 5'-ACGCGT-3'. (**B**) SPR sensorgram of HxIP* **3** with the target 5'-ATCGAT-3' sequence at 100, 200 and 300 nM concentrations of compound. Thick grey lines represent true association and dissociation and thin black lines represent global kinetics fits. (**C**) Steady-state analyses of HxIP* **3** with 5'-ATGCAT-3' (●, solid line), 5'-ATCGAT-3' (▲, dashed line), and 5'-ACGCGT-3' (◆, dotted line) sequences. SPR experiments were run in 10 mM CCL, 1 mM EDTA, 200 mM NaCl, 0.05% P20 (pH 6.5) at 25 °C.

Figure 4: Binding of diamino Hx-polyamides **2** and **3** to the *topo IIα* promoter. Autoradiograms of DNase I footprinting gels, HxI*P **2** (left) and HxIP* **3** (right). The concentrations (μM) used are shown at the top of the gel. G+A represents a formic acid-piperidine marker specific for purines. The positions of the ICB1, ICB2, ICB3 and the target sequence are indicated.

Figure 5: Inhibition of NF-Y binding to ICB2 by the diamino Hx-polyamides. Electrophoretic mobility shift assays (EMSAs) using a radiolabelled oligonucleotide containing the ICB2 and target sequence 5'-TACGAT-3' were pre-incubated with increasing concentrations of polyamide **2** or **3** for 1 h at room temperature prior to addition of the NIH3T3 nuclear extract. 0, control reaction containing the oligonucleotide and nuclear extract without polyamide; C, reaction in the presence of an excess of unlabelled competitor oligonucleotide of the same sequence as the control reaction; M, reaction in the presence of an excess of unlabelled oligonucleotide with a mutation to the ICB2 motif. (**A**) Comparative analysis of the inhibitory effects of HxI*P **2** and HxIP* **3** on the binding of NF-Y containing protein complexes to the ICB2. (**B**) EMSA showing

HxIP* **3** is able to displace NF-Y already bound to the ICB2 when the radiolabelled oligonucleotide is pre-incubated with the nuclear extract prior to the addition of the polyamide. (**C**) Supershift analysis using an anti-NF-YA antibody confirmed the presence of NF-Y in the protein complex bound to the radiolabelled oligonucleotide.

Figure 6: Visualisation of HxI*P **2** and HxIP* **3** nuclear localisation. A549 (top panels) and NIH3T3 (bottom panels) cells were treated with the indicated concentrations of HxI*P (left) or HxIP* (right) for 24 h, washed with PBS, and fixed with 2% paraformaldehyde (PFA). They were subsequently permeabilised and the nuclei were stained with propidium iodide (PI) before confocal microscopy imaging. The composite image presents the superimposed overlay of diamino Hx-polyamide fluorescence and the PI fluorescence. No polyamide fluorescent signal was detected in the control, untreated cells under the same observation settings.

Figure 7: Effect of the diamino HxIP derivatives on topo IIα expression in confluent A549 cells. (**A**) Quantification of topo IIα mRNA levels via RT-PCR analysis. Cells were maintained at confluency before 18 h treatment with increasing concentrations of HxI*P **2** or HxIP* **3**. mRNA levels are compared relative to untreated confluent A549 cells (CON). Error bars represent the SEM from three biological replicates. Statistical significance was calculated using one-way ANOVA analysis (* $p < 0.05$). (**B**) Immunoblot analysis of confluent A549 nuclear extracts probed with topo IIα antibody following 18 h treatment with HxI*P **2** or HxIP* **3**. Lamin is shown as a loading control.

Figure 8: Potentiation of topo II poison induced-cytotoxicity by HxIP*. A549 cells were pre-treated with the indicated concentrations of HxIP* **3** for 24 h and exposed to etoposide (50 μM) for 2 h. After 24 h in drug free medium, they were analysed for γH2AX levels by confocal microscopy and immunoblotting. (**A**) Representative images of A549 cells. (**B**) Number of H2AX foci per nuclei for the indicated treatment combinations, as quantified by CellProfiler Software. (**C**) Immunoblot analysis of the nuclear levels of γH2AX and topo IIα, 24 h after pre-treatment

with HxIP* for 24 h, treatment with etoposide for 2 h and their combination. (**D**) Confluent A549 cells were treated with HxIP* (2 and 5 μM) or etoposide (400 μM) and HxIP*-etoposide combination, where 6 h etoposide exposure followed 24 h pre-treatment with HxIP*. Cell viability (%) was measured using the CellTiter-Glo® assay. Luminescence values are normalised to those of the untreated control. (**E**) Viability was also assessed after 6 h treatment with doxorubicin (75 μM) and HxIP*-doxorubicin combinations, where 6 h exposure followed 24 h HxIP* pre-treatment. All data are represented as mean ± SEM (n=3). Statistical significance was calculated using one-way ANOVA analysis (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).