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Binding of f-PIP, a pyrrole- and imidazole-containing triamide, to the inverted CCAAT box-2 of the topoisomerase IIα promoter and modulation of gene expression in cells

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Abstract—An *N*-formamido pyrrole- and imidazole-containing triamide (f-PIP) has been shown by DNase I footprinting, SPR, and CD studies to bind as a stacked dimer to its cognate sequences: 5′-TACGAT-3′ (5′-flank of the inverted CCAAT box-2 of the human topoisomerase IIα promoter) and 5′-ATCGAT-3′. A gel shift experiment provided evidence for f-PIP to inhibit protein–DNA interaction at the ICB2 site. Western blot studies showed that expression of the topoisomerase IIα gene in confluent NIH 3T3 cells was induced by treatment with f-PIP. The results suggested that the triamide was able to enter the nucleus, interacted with the target site within ICB2, inhibited NF-Y binding, and activated gene expression.

Pyrrole (P)- and imidazole (I)-containing polyamides bind to the minor groove of DNA with sequence specificity,[1–9] and they are potentially useful as gene control agents.[3,5,6] Studies on the development of polyamides have been inspired by the DNA binding properties of distamycin A, a naturally occurring polyamide that prefers A/T-rich sequences. Distamycin A and related polyamides are known to bind tightly as dimers, stacked in an anti-parallel fashion, within the minor groove. The resulting DNA sequence selection is due to stacking of the heterocycles, which interact with the DNA base pairs through a combination of favorable hydrogen bonding, van der Waals forces, and electrostatic interactions. A set of pairing rules has been developed for pyrrole- and imidazole-containing polyamides.[3–5] A stacked P/P pairing recognizes an A/T base pair, a P/I pairing recognizes a C/G base pairing, an I/P recognizes a G/C base pair, and an I/I pairing selects for a T/G mismatch.[10,11] The pairing rules have provided an opportunity for the rational design of compounds capable of targeting predetermined DNA sequences.

Topoisomerase IIα (topoIIα) is an enzyme essential for cell function, including DNA replication and mitosis.[12] It is a target for anticancer agents, including etoposide and doxorubicin.[12,13] Confluent cancer cells down-regulate topoIIα expression, making these cells resistant to therapy by these drugs.[12–15] The promoter region of the topoIIα gene consists of five inverted CCAAT boxes (ICBs);[13] binding of nuclear factor-Y (NF-Y) to ICB2 is responsible for inhibiting gene expression at confluence. The DNA sequence of ICB2 including its 5′-flanking sequence is 5′-TACG**ATTGG**T-3′ (ICB2 is indicated in bold).[13,15] Consequently, ligands designed to target the ICB2 site and block NF-Y from binding should upregulate topoIIα expression in confluent cells, thereby sensitizing these cells to topoIIα acting drugs.[15]

As part of our overall strategy for the design of compounds capable of inhibiting NF-Y from binding to

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ICB, JH-37, a hairpin polyamide depicted in Figure 1, was developed and previously reported.[16] It was found to bind selectively to 5′-TTGGT-3′, which is embedded within the 3′-flanking regions of ICB2 and ICB3.[16] DNase I footprinting and biophysical analysis confirmed the preferential binding of JH-37 to ICB2 and ICB3. However, it was also found to bind the 3′-flank of ICB1, which contained a 5′-TTGGC-3′ site.

Even though JH-37 has demonstrated selectivity for binding to ICB sites, there is a need to design polyamide structures that are simpler and smaller, while improving sequence specificity. It is becoming apparent that even though hairpin polyamides demonstrate excellent sequence selectivity, they often have poor solubility in water and cell culture media. Hairpin polyamides are also taken up poorly by cells, especially into the nucleus.[17]

This paper details the results from an investigation into the question of whether simple polyamides that bind as stacked dimers to their target sequences in the minor groove could elicit biological activity. An advantage of using small polyamides is that they should more readily enter cells and concentrate in the nucleus.[18] For example, a dicationic furamidine analog has been shown by fluorescence microscopy to enter cells and localize in the nucleus. Furamidine is presumed to bind as a stacked dimer in the minor groove of ATGA sites.[19]

f-PIP, a *N*-formamido-triamide shown in Figure 1, was selected for this study.[20] According to the pairing rules,[4,5] it should recognize 5′-ATCGAT-3′ as well as 5′-TACGAT-3′, which is found in the 5′-flank of ICB2. Both are cognate sequences because the stacked P/P pairing could recognize either an A/T or T/A base pair (Fig. 2). The affinity of f-PIP for binding to the 5′-ATCGAT-3′ sequence was determined to be $2 \times 10^5\ M^{-1}$ by using surface plasmon resonance.[20] In this communication, the sequence selectivity of f-PIP and its ability to induce the expression of the topoIIα gene in confluent cells are reported.

DNase I footprinting studies of f-PIP on a fragment of the topoIIα promoter were used to ascertain sequence selectivity. The autoradiogram given in Figure 3 shows that a footprint at the 5′-TACGAT-3′ site (5′-flank of ICB2) begins to appear at a concentration of 10 μM and by 30 μM a clear footprint had formed.

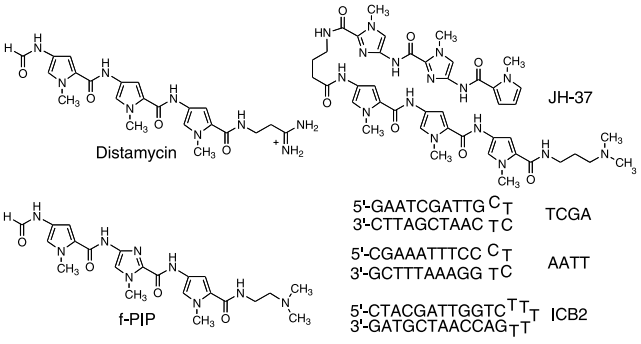

Figure 1. Structures of distamycinA, JH-37, and f-PIP. The sequences of the hairpin oligonucleotides used in this study are also given.

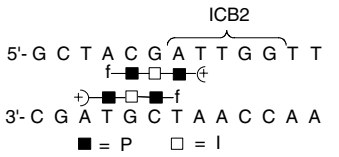

Figure 2. Binding of f-PIP (**2**) to the 5′-TACGAT-3′ site on the 5′-flank of ICB2.

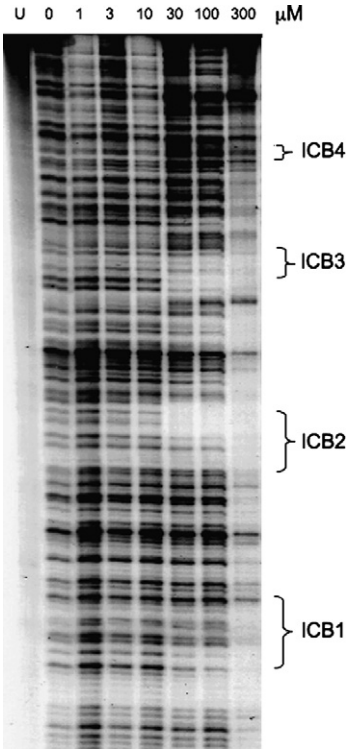

Figure 3. DNase I footprinting of f-PIP using a 5′-^{32}P-radiolabeled probe corresponding to the topoIIα promoter. The ICB1, ICB2, ICB3, and ICB4 sites are indicated. ICB1, 5′-CAGGG*ATTGG*CTGG-3′; ICB2, 5′-CTACG*ATTGG*TTCTT-3′; ICB3, 5′-ACCTG*ATTGG*TTTAT-3′; ICB4, 5′-TTCTC*ATTGG*CCAGA-3′.

A weak footprint for the AT-rich sequence on the 3′-flank of ICB3 did not become apparent until 30 μM. It is worthy to note that f-PIP did not protect ICB1 and ICB4 from DNase I cleavage even at 100 μM. This result represents an improvement in selectivity when compared to JH-37, which showed binding to sequences within the ICB1, 2, and 3 sites.[16]

The DNA binding properties of f-PIP were further assessed by circular dichroism studies,[6–8,20] and the results are given in Figure 4. Titration of f-PIP to the cognate oligonucleotides (ICB2 and TCGA, Figure 4A and B, respectively) produced a DNA-induced band at 330 nm, suggesting that the triamide was bound within the minor groove.[21] It is worthy to note that a weak DNA-induced band was observed for the AT-rich hairpin oligonucleotide (Fig. 4C). This result was not surprising because f-PIP had demonstrated binding to an

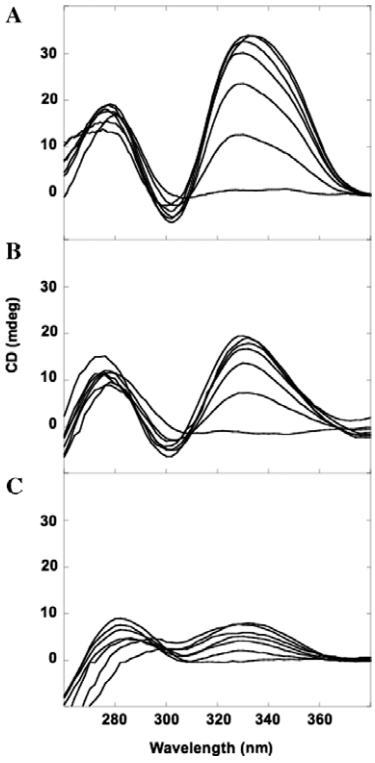

Figure 4. CD titration studies of f-PIP with ICB2 (A), TCGA (B), and AAATTT (C). The studies were performed on an OLIS spectropolarimeter, using a 1 mm pathlength cuvette. Experiments were carried out using a 10 mM phosphate buffer, containing 50 mM Na^+, and 1 mM EDTA, pH 6.2, and was titrated with 1 mol equivalents of the polyamide f-PIP, past the point of saturation. ICB2, 5′-CTA CG*A TTG GTC* TTTTT GAC CAA TCG TAG; TCGA, 5′-GAA *TCG A*TT G CTCT C AAT CGA TTC; AAATTT, 5′-CG*A AAT TT*C C CTCT G GAA ATT TCG.

AT-rich sequence in the footprinting analysis (Fig. 3). It has been reported that single imidazole-containing polyamides can tolerate AT-rich sequences.[22] The appearance of an isodichroic point in the overlaid spectra further indicated that the compound was binding to the DNA through one mechanism, presumably as a stacked dimer.[20]

Electrophoretic mobility shift assays (EMSA) can provide direct evidence for the ability of small molecules to affect the binding of transcription factors to target DNA sequences.[15] EMSA studies for the binding of f-PIP to 5′-^{32}P radiolabeled ICB1 and ICB2-containing DNA fragments were conducted and the results are given in Figure 5. At a concentration of 50 μM, f-PIP completely inhibited protein binding present in nuclear extracts of confluent cells, to the ICB2 oligonucleotide. The complex includes NF-Y as demonstrated by antibody supershift experiments (data not shown). The inhibition is presumably due to binding of f-PIP to the 5′-TACGAT-3′ sequence, which partially overlaps the ICB2 site. At a similar concentration, f-PIP did not displace NF-Y from binding to ICB1, which was consistent with the inability of f-PIP to bind to ICB1 according to the footprinting studies (Fig. 3). For comparison, JH-37 inhibited the binding of NF-Y to ICB2 at 1–5 μM (data not shown).

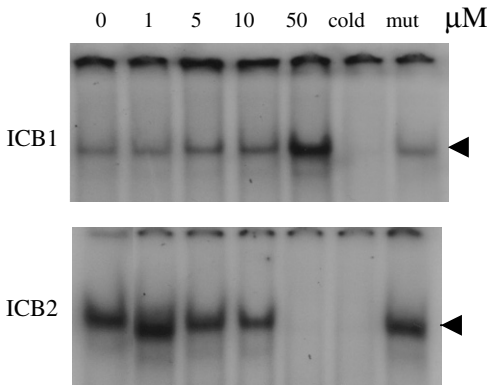

Figure 5. EMSA studies on f-PIP using oligonucleotides containing ICB1 and ICB2 sequences present on the promoter of topo-IIα. The oligonucleotides were incubated with f-PIP (concentrations ranging from 0 to 50 μM) for 2 h prior to incubation with nuclear extracts from cultured confluent NIH 3T3 cells. Cold and mut lanes represent reactions carried out using unlabeled and mutated oligonucleotides, respectively.

In order to demonstrate whether stacked dimers of polyamides could be developed as cellular gene control agents, the ability of f-PIP to enter the nucleus of cells and affect gene expression was investigated.[15] Confluent NIH 3T3 cells were incubated with 10 μM f-PIP for 4, 6, and 24 h. Western blot analyses of treated cells were performed and the results are given in Figure 6 compared to results obtained with JH-37.

The results confirm that topoIIα protein expression in confluent cells was reduced as compared to exponentially growing cells.[13] However, as indicated in Figure 6B, JH-37 was found to induce topoIIα protein expression after 24 h of exposure. f-PIP treatment showed increased level of topoIIα protein expression after only 4 h of exposure which increased with duration of exposure (Fig. 6A), even though its binding constant to ICB2 was lower than JH-37 ($3 \times 10^7\ M^{-1}$).[16] This suggested that the smaller f-PIP triamide could more effectively enter cells and concentrate in the nucleus than the larger hairpin molecule (Fig. 6B). The results also show that, like JH-37, f-PIP was able to induce the expression of topoIIα by presumably binding as a stacked dimer on the 5′-TACGAT-3′ sequence in the 5′-flank of ICB2 and

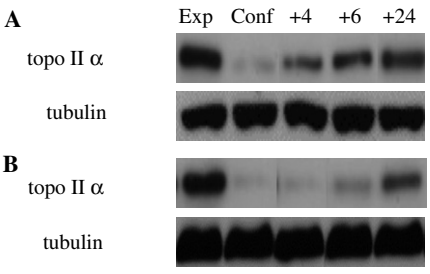

Figure 6. NIH 3T3 cells were either exponentially growing (Exp) or maintained at confluence for 96 h (Conf). Confluent cells were treated with 10 μM f-PIP (A) or JH-37 (B), and Western blot analysis was carried out on samples collected at +4, +6, and +24 h post-treatment as indicated. The IHIC8 rabbit polyclonal topo-IIα antibody was used for Western blotting, and tubulin is shown as a loading control.

preventing binding of NF-Y. In conclusion, and to our knowledge, this report details the first example of a simple triamide analog of distamycin, capable of binding as a stacked dimer and affecting gene expression in cells.

Acknowledgments

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