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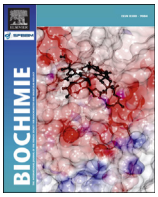

Research paper

Binding studies of a large antiviral polyamide to a natural HPV sequence

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ABSTRACT

PA1 is a large hairpin polyamide (dImPyPy-β-PyPyPy-γ-PyPy-β-PyPyPyPy-β-Ta; Py = pyrrole, Im = imidazole, β = beta alanine) that targets the sequence 5′-WWGWWWWWWW-3′ (W = A or T) and is effective in eliminating HPV16 in cell culture (Edwards, T. G., Koeller, K. J., Slomczynska, U., Fok, K., Helmus, M., Bashkin, J. K., Fisher, C., *Antiviral Res.* 91 (2011) 177–186). Described here are its DNA binding properties toward a natural DNA, a 523 bp portion of HPV16 (2150–2672) containing three predicted perfect match sites. Strategies for obtaining binding data on large fragments using capillary electrophoresis are also described. Using an Fe EDTA conjugate of PA1, 19 affinity cleavage (AC) patterns were detected for this fragment. In many cases, there are multiple possible binding sequences (perfect, single and double mismatch sites) consistent with the AC data. Quantitative DNase I footprinting analysis indicates that perfect and most single mismatch sites bind PA1 with K_ds between 0.7 and 4 nM, indicating excellent tolerance for the latter. Double mismatch sites exhibit K_ds between 12 and 62 nM. A large fraction of the accessible sequence is susceptible to PA1 binding, much larger than predicted based on the literature of polyamide-DNA recognition rules.

1. Introduction

N-Methylpyrrole/imidazole-based polyamides (PA) can be considered higher homologs of the natural product distamycin A, a tris(pyrrole) reagent with a cationic C-terminus that binds to the minor groove of AT-rich DNA sequences and which has antitumor and antiviral properties along with high toxicity [1,2]. Polyamides, the binding capabilities of which extend to GC-containing sequences via the incorporation of imidazole building blocks, have found extensive application as sequence-selective DNA binding agents for the control of gene expression [3,4]. Typical modern PAs in use for such experiments employ the hairpin structure in which two polyamide strands are joined by gamma-aminobutyric acid, which can be thought of as a hairpin turn that allows two linked antiparallel polyamide strands to align in the minor groove of DNA and recognize the two DNA strands independently. Other building blocks have been used to construct analogous turns, including (R)-2,4-diaminobutyric acid, its acetamide and more [5–7]. In certain cases the PA strands have been linked at both ends, resulting in a cyclic PA [8–10].

Hairpin polyamides have long been used by Dervan [3,11,12], Sugiyama [6,13], Lee [14–16], Kodadek [17] and others for their ability to recognize DNA sequences via hydrogen bonding interactions in the minor groove [5,9,18–30]. The properties of polyamides have been valuable for controlling gene expression, including the blocking gene-specific transcription factor binding sites and alkylating specific DNA regions [31,32]. Polyamides have also been used to enhance gene transcription [33], and effects have been shown against pathogens and metabolic diseases at the cell culture level. Recently, whole animal efficacy, imaging and pharmacology or toxicology data have been reported for a number of disease targets [4,10,34–39].

The accessibility of PA to nucleosomes has been studied, the use of PA for specific disease states has been fruitful, and the number of *in vivo* studies (for safety and/or efficacy) of PA has been increasing. Adding alkylating functionality to polyamides has allowed them to act directly as gene silencing agents. Uptake of polyamides by cells has been studied and found to vary depending on cell type and PA characteristics in some cases [7,40–44], while in other cases uptake was constant over a wide range of polyamide MW (from 400 to

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4000) [44]. As described in the cited reports, uptake has been enhanced by a variety of means, such as blocking multidrug resistance efflux pumps with Verapamil® or modifying polyamide building blocks at either internal or terminal positions.

Recently, dramatic antiviral effects for human papillomavirus (HPV) were discovered for certain long polyamides that bound 10 or more bp of DNA [45,46]. The active polyamides were designed to bind the dsDNA genome of HPV, an approximately 8 kb circular DNA molecule known as an episome. With IC_{50} values of $\leq$30–100 nM and no measurable cellular toxicity, the active polyamides function by eliminating viral DNA from infected human keratinocytes. Furthermore, the active compounds were shown to restore the phenotype of uninfected cells in keratinocyte tissue culture. Thus, HPV infections manipulate DNA synthesis by host enzymes, forcing suprabasal DNA synthesis to occur for infected keratinocyte tissue cultures; this effect is reversed by addition of properly-designed polyamides. PA1 (Fig. 1) is one of the lead compounds reported in this anti-HPV study, and shows excellent activity against HPV16 and 31 (IC_{50} = 100 and 108 nM). More recently, a detailed report has appeared on the mechanism of action of this class of anti-HPV compounds [46].

The anti-HPV PAs are considerably larger than most reported hairpin polyamides and contain 14–26 rings. Broad-spectrum activity was a surprise because the analogous DNA sequences of the different HPV subtypes are not typically degenerate for polyamide binding. Therefore the origin of primary antiviral activity and broad spectrum activity are both of considerable interest from a range of perspectives, including cellular and biophysical perspectives as well as for the design of more-active second-generation anti-HPV compounds. In light of this broad-spectrum activity, the applicability of polyamide-DNA recognition rules developed mostly for smaller PA molecules (typically 8 rings) was of specific interest for the longer, antiviral polyamides [2,3,11,12,18,19,22,26,27,29,30,47–49]. The use of a large natural sequence target is also important, as it better represents the biological setting than shorter sequences tailored to address specific questions about binding behavior [7]. In this genomic viral DNA setting, what level of detail can be achieved with commonly used assays and approaches? What is the relationship between predicted and observed binding sites? To begin to address this, the binding properties of the large, archetypal anti-HPV polyamide, PA1 (Fig. 1), are therefore characterized here using an HPV16 DNA sequence as the target.

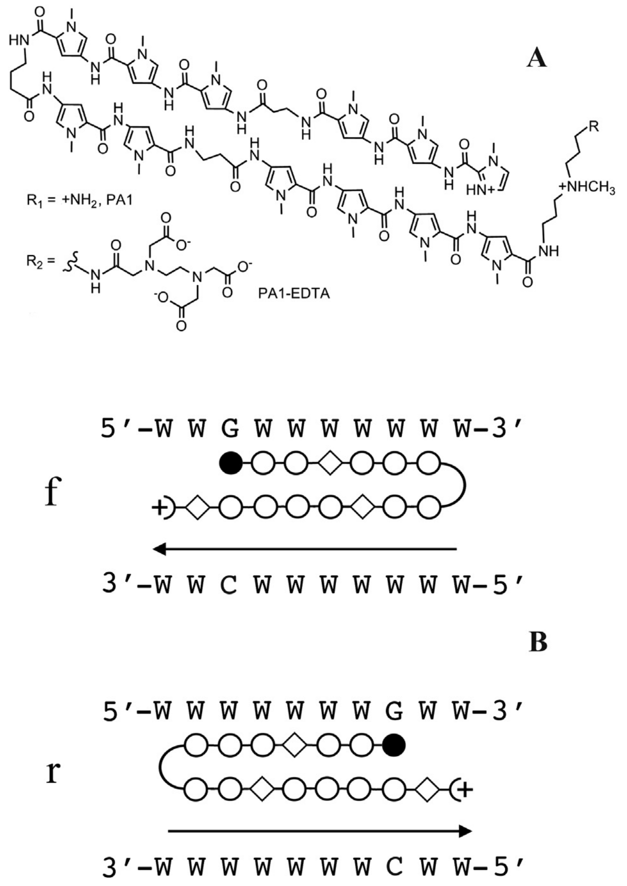

Fig. 1. Structure and predicted recognition sequence for PA1. A, Structure of PA1 and its EDTA conjugate. B, Target sequence for PA1 and alternative binding orientations. Closed circles = imidazole; open circles = pyrrole; open diamond = β alanine W = A/T. The arrowhead indicates the location of the polyamide tail and EDTA group. f refers to the forward binding orientation (amino terminus aligns with 5′ end of target sequence); r refers to the less favored reverse binding orientation (amino terminus aligns with the 3′ end of the target sequence.

2. Materials and methods

2.1. Synthesis

dIm-PyPy-β-PyPyPy-γ-PyPy-β-PyPyPyPy-β-Ta (PA1) was prepared and characterized as previously described [45]. Im = imidazole, dIm = desamino-imidazole, Py = pyrrole, β = beta alanine, γ = γ-aminobutyric acid, and Ta = amino tail at C-terminus formed from $NMe(CH_2CH_2CH_2NH_2)_2$. The EDTA conjugate was prepared as follows: By analogy to the literature [50], to a vigorously-stirred mixture of EDTA dianhydride (23 mg, 0.088 mmol, 20 eq) in N,N-diisopropylethylamine (DIEA) (0.5 mL), DMF (0.25 mL), and DMSO (0.25 mL) at 55 °C was added a mixture of PA1 (10 mg, 0.0044 mmol, 1 eq) in DIEA (0.5 mL) and DMF (0.5 mL) dropwise over a period of 10 min. The reaction mixture was stirred at 55 °C for 20 min. Following addition of 0.1 N NaOH (1.8 mL), the mixture was stirred at 55 °C for an additional 15 min. The bottom layer of the biphasic mixture was removed from the reaction vessel and neutralized by addition of 0.35 M aq. TFA (1 mL). The mixture was diluted with 0.5 mL DMSO and divided into two equal portions, each of which was filtered through 20 μm polyethylene filters. Each portion was further diluted with 0.5 mL DMSO and purified by reversed-phase HPLC [H_2O (0.1% TFA), MeOH (0.1% TFA)] using a Phenomenex Jupiter 250 × 21.2 mm, 4 μm, 90 Å, C_{12} column. Concentration of pooled fractions, followed by lyophilization afforded the PA1-EDTA conjugate (3.8 mg, 33% yield) as a white, fluffy solid: average molecular weight = 2169.27, experimental (ESI) $[M + H]^+$ = 2169.8.

2.2. Footprinting

To generate the 523 bp DNA fragment comprised of the 2150–2672 region of the HPV16 genome (Accession # AF125673), two primers oligomers were ordered from Integrated DNA Technologies, Inc. (Coralville, Iowa) (top strand): 5′-FAM-AT GTG ATA GGG TAG ATG ATG GAG GTG; (bottom strand) 5′-HEX CT CAT ACA CTG GAT TTC CGT TTT CGT CAT ATG G-3′. To generate the smaller duplexes, the following primers were used: top strand FAM-TGG TGC AGC TAA CAC AGG TAA starting at 2291; top strand FAM-CTA CAG TGC CCT GTT GGA AC starting at 2434; bottom strand HEX-CAA CAG GGC ACT GTA GCA TC starting at 2449; bottom strand HEX-CT CAT ACA CTG GAT TTC CGT TTT CGT CAT ATG G starting at 2672.

DNase I footprinting experiments were performed as previously described [49] in TKMC buffer with 10 mM CHAPS. DNA concentrations were adjusted between 200 pM and 10 nM as needed. A small range of DNase I units (0.01–0.02 U in a 250 μL reaction) and a

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