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Sequence-selective binding of C8-conjugated pyrrolobenzodiazepines (PBDs) to DNA

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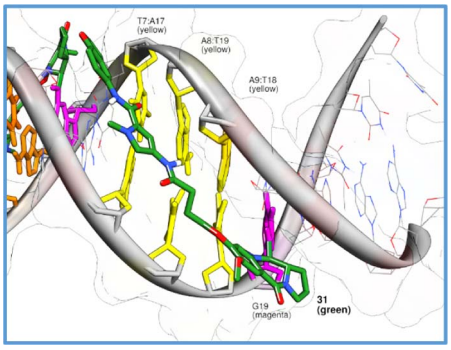
HIGHLIGHTS

- C8-conjugates of pyrrolobenzodiazepines with benzofused rings modulate sequence-selective DNA binding.
- The conjugates bind best to guanines flanked by A/T-rich sequences.
- Bi- and tri-phasic melting curves show that 14-mer oligonucleotides can bind more than one ligand.

GRAPHICAL ABSTRACT



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ABSTRACT

DNA footprinting and melting experiments have been used to examine the sequence-specific binding of C8-conjugates of pyrrolobenzodiazepines (PBDs) and benzofused rings including benzothiophene and benzofuran, which are attached using pyrrole- or imidazole-containing linkers. The conjugates modulate the covalent attachment points of the PBDs, so that they bind best to guanines flanked by A/T-rich sequences on either the 5′- or 3′-side. The linker affects the binding, and pyrrole produces larger changes than imidazole. Melting studies with 14-mer oligonucleotide duplexes confirm covalent attachment of the conjugates, which show a different selectivity to anthramycin and reveal that more than one ligand molecule can bind to each duplex.

1. Introduction

Pyrrolobenzodiazepines [1] are sequence selective DNA minor groove binding ligands, of which anthramycin (**1**; Fig. 1) was the first compound to be isolated in the 1960s from cultures of *Streptomyces* [2]. These compounds have received considerable attention because of their potent antitumour activity, which results from covalent binding of the C11 position of the diazepine ring to the 2-amino group of a guanine [3,4]. Naturally occurring PBDs such as anthramycin, tomaymycin, neothramycin and sibiromycin form adducts that span three base pairs, with guanine in the central position [5,6], and their preferred binding site is 5′-AGA-3′ [7], although more recent data suggest that they have a kinetic preference for 5′-YGR-3′ sequences [8] (R = purine, Y = pyrimidine). Synthetic PBD monomers with C2 (pyrrolidine ring)-aryl

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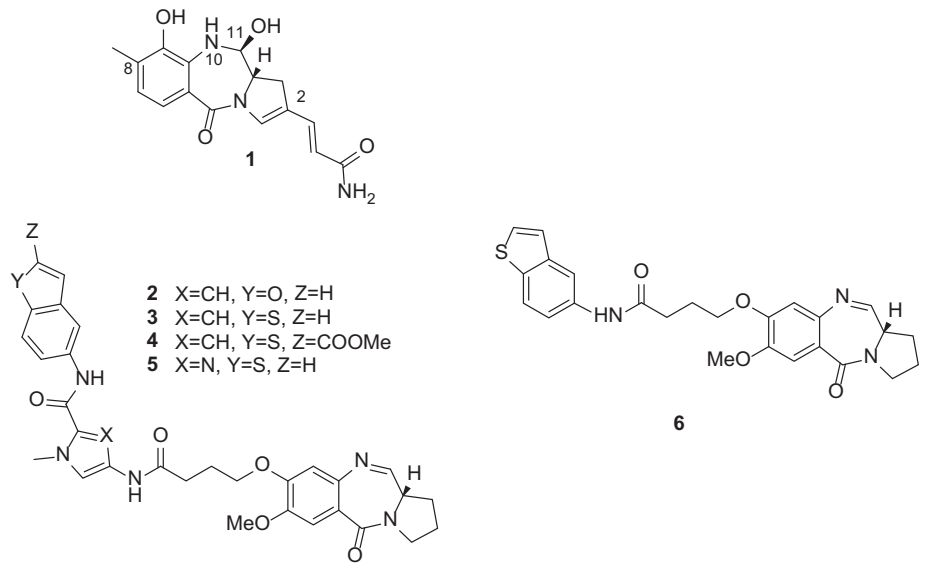

Fig. 1. Structures of PBD-polyamide conjugates. (**1** = anthramycin).

substitution exhibit nanomolar to picomolar cytotoxic potency against a number of tumour cell lines [9]. In an attempt to enhance their binding selectivity, bis-PBDs have been developed in which two PBD monomers are joined by a suitable linker and SJG-136, which forms interstrand cross-links at 5′-RGATCY-3′ as well as intrastrand cross-links and mono-adducts [10,11], has reached Phase II clinical trial [12,13]. Another approach for targeting longer DNA sequences is to prepare PBD C8-conjugates, in which heterocyclic rings such as pyrrole and imidazole are linked to the PBD anthranilate ring through amide bonds [14–17].

Polyamides composed of pyrrole and imidazole heterocycles are able to form antiparallel dimers in the DNA minor groove and are widely known for their ability to recognize AT and TA base pairs with pyrrole-pyrrole pairs, and CG and GC with pyrrole-imidazole and imidazole-pyrrole pairs, respectively [18–20]. The conjugation of a PBD with a polyamide requires a suitable linker between these two entities. By comparing the binding energy of different linkers, a trimethylene group has been shown to provide a good isohelical fit in the DNA minor groove [15]. A conjugate of a PBD linked with a polyamide composed of two pyrroles was found to recognise the sequence (CCATT) in the promoter of DNA Topo IIα and to inhibit the binding of NF-γ, which is involved in cell cycle progression [17]. The combination of pyrrole and thiazole showed better DNA binding affinity than di-pyrroles [16], while addition of an imidazole offered superior sequence selectivity [14]. In addition, several other PBD conjugates have shown significant antibacterial [21] and antitubercular activity [22]. More recently, members of the PBD family have been developed as cytotoxic payloads for attachment to antibodies to form antibody-drug conjugates (ADCs) and a number of these are presently in the clinic for the treatment of various leukaemias and lung cancer [1].

There has been significant interest in trying to understand the molecular basis of the selectivity of known DNA-targeting agents with a view to designing novel molecules targeted to specific sequences within genes to allow selective modulation of expression for both research and therapeutic purposes. We previously reported a series of C8-linked pyrrolobenzodiazepine (PBD)-poly(N-methylpyrrole) conjugates (*e.g.*, GWL-78) which demonstrated the synergistic effect of joining a GC-specific PBD unit to an AT-recognizing polypyrrole fragments [15]. More recently we identified 4-(1-methyl-1H-pyrrol-3-yl)benzenamine (MPB) as a GC-selective building block (Fig. 1) and coupling the MPB motif to PBDs through the C8-position resulted in molecule with sub-nanomolar to femtomolar IC50 across 65 cell lines tested [23]. As part of our aim to develop pyrrolobenzodiazepine based selective anti-tumour compounds, and to understand the role of benzofused building blocks in sequence selectivity, we linked benzofuran, benzothiophene or benzopyrrole (indole) groups to the unsubstituted pyrrolobenzodiazepine core through a C4 linker. The benzofused groups were either directly linked to the PBD core through the C4 linker or separated by a further pyrrole/imidazole heterocycle to assess the impact of pyrrole/imidazole heterocycles on the DNA stabilisation potential of PBD-benzofused conjugates.

In this paper, we describe the DNA-binding properties of five C8-PBD-polyamide conjugates with benzofused rings (**2–6**; Fig. 1), and have examined their DNA binding sequence selectivity by DNase I footprinting and fluorescence melting.

2. Materials and methods

2.1. C8-PBD-conjugates

The structures of the C8-PBD-conjugates used in this work are shown in Fig. 1. Their synthesis will be reported elsewhere and the purity data are presented in the Supplementary material. The compounds were dissolved in dimethylsulfoxide at a concentration of 10 mM and stored at − 20 °C. These were diluted to working concentrations in 10 mM Tris-HCl pH 7.5 containing 10 mM NaCl immediately before use.

2.2. DNase I footprinting

Footprinting reactions were performed as previously described [24] using the DNA fragments HexA and HexB, which together contain all 64 symmetrical hexanucleotide sequences [25], and MS1 that contains all possible 134 tetranucleotide sequences [26]. The DNA fragments were obtained by cutting the parent plasmids with HindIII and SacI (for HexA and MS1) or EcoRI and PstI (for HexB), and were labelled at the 3′-end of the HindIII or EcoRI sites with [α-^{32}P]dATP using reverse transcriptase or exo-Klenow fragment. After gel purification, the radiolabelled DNA was dissolved in 10 mM Tris-HCl pH 7.5 containing 0.1 mM EDTA, at a concentration of about 10 c.p.s per μL as determined on a hand held Geiger counter. 1.5 μL of radiolabelled DNA was mixed with 1.5 μL ligand that had been freshly diluted in 10 mM Tris-HCl pH 7.5, containing 10 mM NaCl. The complexes were left to equilibrate for at least 12 h before digesting with 2 μL DNase I (final concentration about 0.01 units/mL). The reactions were stopped after 1 min by adding 4 μL of formamide containing 10 mM EDTA and bromophenol blue (0.1% w/v). The samples were then heated at 100 °C for 3 min