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DNA sequence-selective monoheterocyclic analog of Hoechst 33258: cytotoxicity and antiparasitic properties

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Abstract

The biophysical and biological evaluations of DNA minor groove binding AT sequence selective benzimidazole analogs of Hoechst 33258 which contain a *p*-anisyl, a *p*-[bis(2-chloroethyl)amino]phenyl or a *p*-anisyl and an amidine moiety are discussed. The preference for all three compounds for the 5′-AAATTT-3′ sequence was ascertained by thermal denaturation and circular dichroism studies. The mustard-containing compound **4** was found to be more cytotoxic against murine cancer cells grown in culture than the non-mustard containing compound. DNA alkylation was not necessary for anti-*Leishmanial* activity.

Keywords: circular dichroism; cytotoxicity; DNA; Hoechst 33258; thermal denaturation.

Introduction

There is currently a wealth of interest in the study and development of low-molecular weight sequence-selective agents capable of binding to double-stranded DNA. Such agents are often based on chromosomal staining agents and natural products that have been extensively investigated for their selective binding to minor groove DNA. Such an example is Hoechst 33258, **1**, a DNA minor groove binder that is known for recognizing AT rich sequences (Harshman and Dervan, 1985; Pjura et al., 1987; Teng et al., 1988; Parkinson et al., 1989, 1990). After extensive clinical evaluation, it was rendered clinically useless owing to its low activity and high toxicity (Lomax and Narayanen, 1986). Its benzoyl mustard derivative of distamycin, tallimustine (or FCE 24517), **2**, despite showing promising activity against a wide spectrum of solid tumors including a handful of murine (Pezzoni et al., 1991; Capolongo et al., 1993) and DNA alkylation-resistant tumors (Arcamone et al., 1989), was also discontinued owing to severe myelotoxicity (Arcamone et al., 1989). Analogous to the distamycin derivatives, we had previously envisioned that the incorporation of nitrogen mustard moieties into monoheterocyclic bisbenzimide or Hoechst 33258 analogs might improve their anticancer properties. With this information in hand, we have previously synthesized analogs **3** and **4**, and shown for the importance of the mustard moiety in **4** in various aspects of selective binding (to AT-rich DNA sequences within the minor groove) and cytotoxic properties, namely DNA crosslinking ability (agarose gel assay) and activity against the leukemic K562 cell line (Lee et al., 1993).

Herein we present further biological and biophysical characterization in the form of activity against leukemic and murine cancer cell lines (L1210 and B16) along with circular dichroism (CD) and thermal melting studies. In addition, we were interested to ascertain the effect of replacing the bulky cationic group in **3** for a planar amidine group against the growth of cancer cells and parasites. Studies by other groups have shown this significantly enhances DNA interaction affinity in several benzimidazole-amidine systems (Czarny et al., 1995; Clark et al., 1997; Bostock-Smith et al., 2001). As a result, we chose to synthesize **5** to decipher any differing biological and biophysical characteristics (and ultimately the mechanism of action) between a conventional chemotherapeutic alkylating agent such as **4** and a typical monoheterocyclic benzimidazole monocation system targeted towards leishmaniasis (Tanious et al., 2004; Wilson et al., 2005; Mayence et al., 2008; Wilson et al., 2008).

Results and discussion

Compounds **3** (Lee et al., 1993), **4** (Lee et al., 1993) (the characterization data for compounds **3** and **4** was in agreement with that of the data previously reported in Lee et al., 1993) and **6** (the precursor for **5**), were synthesized by reaction of the relevant 1,2-phenylenediamine with an aromatic aldehyde in the presence of refluxing nitrobenzene (Bathini and Lown, 1990). Benzimidazole **6** was synthesized in 50% yield as a yellow solid. The conversion of **6** to the amidinium salt **5** was achieved using a modified literature procedure (Jendralla et al., 1996; Judkins et al., 1996). Treatment of the nitrile **6** with hydroxylamine hydrochloride in refluxing methanol in the presence of excess triethylamine furnished the resulting crystalline amidoxime. Acetylation of the amidoxime in the presence of acetic anhydride in glacial acetic acid gave the acetylated amidoxime (Figure 1), which was immediately hydrogenated in methanolic AcOH solution to give the amidinium salt **5** as a tan solid in an overall yield of 53% from intermediate **6**.

The anticancer *in vitro* cytotoxicity of compounds **3**, **4** and **5**, against the growth of B16 (murine melanoma) and L1210 (murine leukemia) cells was evaluated using a 72-h

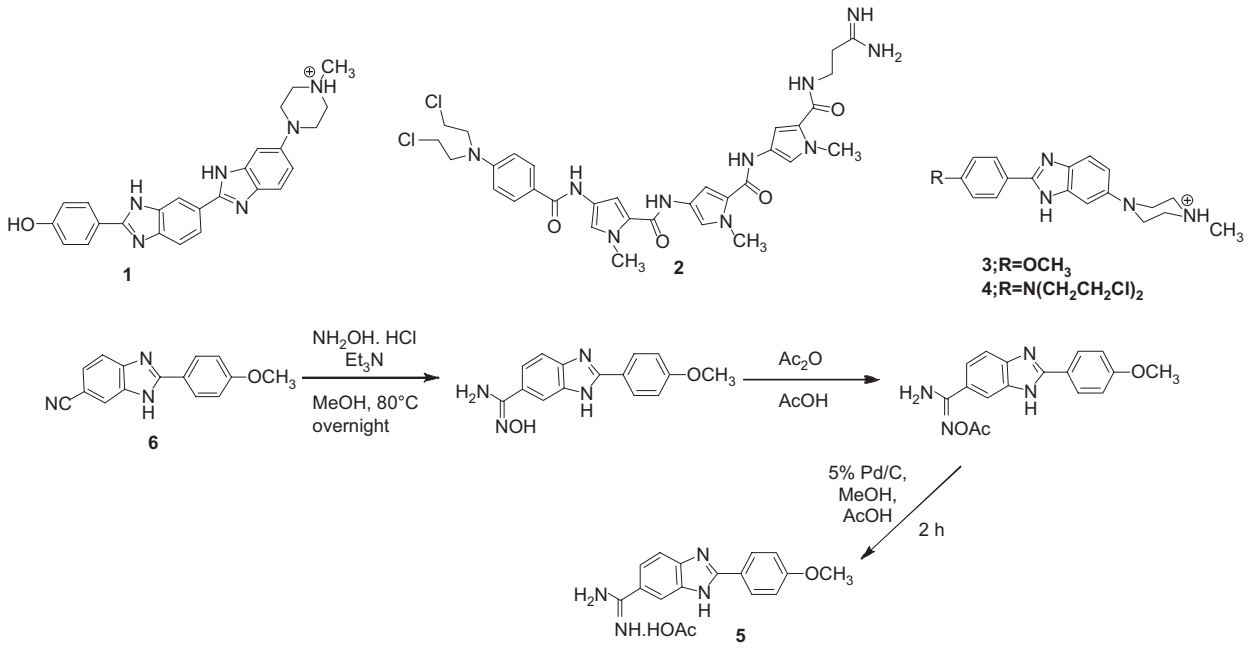

Figure 1 Structures of bisbenzimide or Hoechst 33258 (**1**), tallimustine (**2**), benzimidazole analogs **3** and **4**, and the synthesis of amidine **5** from nitrile **6**.

continuous exposure MTT assay (LeBlanc et al., 2005). The concentrations of compounds that inhibited the growth of tumor cells by 50% relative to an untreated control or IC_{50} values are shown in Table 1. Compound **4** containing the full mustard moiety showed promising cytotoxicity towards both B16 and L1210 cell lines in the submicromolar range (IC_{50} values 3–4 μM). By comparison, the non-mustard counterpart **3** showed virtually no cytotoxicity (IC_{50} >100 μM, Table 1). Although the amidine **5** showed no cytotoxicity against the B16 cell line, a good level of cytotoxicity against L1210 was observed (IC_{50}=6.3 μM, Table 1) clearly demonstrating that **5** exerts its cytotoxicity towards L1210 cells via a different mechanism than that of **4**, although to a lesser extent. The antiparasitic *in vitro* activity of **3**–**5** against *Leishmania tarentolae* (cultured in BHI media) was assessed using an MTT assay with the cytotoxicity of the compounds evaluated after 72 h of continuous exposure. In addition, the compounds were dissolved in media containing 0.03–0.5% DMSO which has been previously shown not to effect the growth of the parasites (Desta et al., 2010). Compounds **3** and **4** showed a moderate level of cytotoxicity towards *L. tarentolae* (IC_{50}=63 and 78 μM, respectively, Table 1), whereas **5** showed virtually no cytotoxicity against the parasites.

Table 1 DNA melts [ΔT_M (°C)] and IC_{50} values of compounds **3**–**5** against murine cancer cells (B16 and L1210) and against the parasite *Leishmania tarentolae*.

Compound	ΔT_M (°C)		IC_{50} (μM)		
	A_3T_3–10	ACGCGT	B16	L1210	*L. tarentolae*
3	9	0	>100	>100	63
4	8	0	3.3	3.4	78
5	5	0	>100	6.3	>100

The binding of **3**–**5** to minor groove DNA was tested using the cognate DNA sequence 5′-AAATTT-3′ (A_3T_3–10) and the non-cognate 5′-ACGCGT-3′ in thermal denaturation experiments. Consistent with reported DNA binding properties of Hoechst analogs (Beerman et al., 1992), the results given in Table 1 confirmed that all three compounds were able to stabilize AT-rich DNA as opposed to GC-rich DNA as shown by the ΔT_M values derived from the differences in melting temperature of the DNA-polyamide complexes and duplex DNA alone. CD spectroscopy was also used to verify the binding of **3**–**5** in the minor groove of double-stranded DNA sequences mentioned. CD assays were conducted by titrating each ligand with DNA solutions comprising the DNA sequences tested in the thermal denaturation studies. These studies showed the appearance of weak induced CD bands at ~330 nm along with the appearance of an isodichroic point in the CD overlaid spectra (data not shown) for **3**–**5** with 5′-AAATTT-3′ indicating some degree of binding. By comparison, no binding was observed for **3**–**5** with 5′-ACGCGT-3′ as indicated by the absence of an induced CD band. Overall, the CD data corroborates the thermal melt data presented.

In conclusion, three monoheterocyclic molecules structurally related to Hoechst 33258 were synthesized. The presence of a nitrogen mustard on **4** confers its ability to be cytotoxic against cancer cells in culture, presumably owing to sequence-selective alkylation at AT-rich sites. It is noteworthy that, contrary to bisbenzamidines reported in the literature (Wilson et al., 2008), compound **5** does not exhibit significant binding

(as shown by the biophysical data) to DNA. It is, however, worthy to note that the antiparasitic activity does not have to be coupled to DNA alkylation, which causes DNA damage and toxicity. However, the results suggest that the antiparasitic activity is connected to DNA binding affinity, measured by the ΔT_M values. This finding is consistent with the DNA binding and biological activity of published diamidines (Wilson et al., 2008).

Experimental

Each of the synthesized compounds were subjected to *in vitro* cytotoxicity screening as described previously (LeBlanc et al., 2005; Desta et al., 2010). All compounds were characterized by melting point, IR, elemental analysis and ^{1}H NMR. Melting points were recorded on a Gallenkamp apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian 400 MHz spectrometer using TMS as an internal standard (δ in ppm). Thermal denaturation (T_M): DNA oligomers were purchased from Operon (Huntsville, AL) with the following sequences: A_3T_3–10; 5′-CGA AAT TTC CCT CTG GAA ATT TCG-3′ and ACG CGT; 5′-GAA CGC GTC GCT CTC GAC GCG TTC-3′. Thermal denaturation studies were performed using published procedures (Mackay et al., 2008). Experiments for **3**–**5** were performed at a concentration of 3 μM ligand and 1 μM DNA. All experiments were run in PO_{40} buffer. Oligonucleotide samples were reannealed prior to denaturation studies by heating at 70°C for 1 min and then cooling to room temperature. Heating runs were typically performed between 25°C and 95°C, with a heating rate of 0.5°C min^{-1} while continuously monitoring the absorbance at 260 nm (digitally sampled at 200 ms intervals). All melts were performed in 10-mm path length quartz cells. T_M values were determined as the maximum of the first derivative. CD studies: these studies were also performed using previously reported procedures (Mackay et al., 2008) and were conducted at ambient temperature in a 1-mm path length quartz cell using PO_{40} buffer. Buffer and stock DNA were added to the cuvette to give a final DNA concentration of 9 μM. Each polyamide (in 500 μl in double-distilled H_2O) was titrated in 1 molar equivalent into the relevant DNA (160 μl of 9 μM DNA) until saturation was achieved. Each run was performed over 400–220 nm. The CD response at the λ_{max} of the induced peak was plotted against the molar ratio of ligand:DNA.

Procedure for the preparation of compound 5

Et_3N (0.2 ml, 1.61 mmol) and hydroxylamine hydrochloride (112 mg, 1.61 mmol) were added to a solution of **6** (100 mg, 0.4 mmol) in MeOH (10 ml). The resulting solution was refluxed for 12 h after which TLC analysis showed the complete disappearance of the starting material. The MeOH was evaporated off leaving a crude white solid, which was immediately taken up in glacial acetic acid (4 ml). Ac_2O (0.14 ml, 1.53 mol) was added and the resulting solution refluxed at 100°C for 3 h after which TLC analysis showed the disappearance of the amidoxime starting material. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure using a Kugelrohr apparatus to give a tan residue which was taken up in distilled water (4 ml), washed with DCM (4 ml), dried over magnesium sulfate and evaporated to dryness to give the acetylated amidoxime. A 100-ml round bottom flask containing the acetylated amidoxime (0.21 g) was immediately charged with 10% Pd/C (125 mg), glacial acetic acid (0.5 ml) and MeOH (10 ml). The resulting mixture was degassed and stirred under H_2 for 2 h after which the reaction mixture was filtered over a pad of celite and co-evaporated three times with dry DCM (5 ml) followed by concentration under reduced pressure to give **5** as a tan solid (70 mg, 53%); m.p. 145–147°C; R_F [MeOH: $CHCl_3$ (50:50)] 0.34; υ cm^{-1} (neat) 3400, 2900, 2963, 1651, 1607, 1443, 1254, 1254, 1030, 919 and 730; ^{1}H NMR (400 MHz, CD_3OD) 8.51 (2 H, s, br), 8.10 (2 H, d, *J* 8 Hz), 8.08 (1 H, s), 7.75 (1 H, d, *J* 8 Hz), 7.68 (1 H, d, *J* 8 Hz), 7.13 (2 H, d, *J* 8 Hz), 4.65 (1 H, s, br), 3.89 (3 H, s) and 1.96 (3 H, s); LRMS (ES$^+$) *m/z* ([M+H$^+$] $C_{15}H_{15}N_4O^+$) 267 (50%), 250 (7%), 175 (30%), 155 (100%) and 134 (18%); HRMS found $C_{15}H_{15}N_4O^+$ 267.1235; $C_{15}H_{15}N_4O^+$, requires 267.1246.

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