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Original article Design of novel bis-benzimidazole derivatives as DNA minor groove binding agents



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ABSTRACT

A new series of bis-benzimidazole derivatives were designed and synthesized. *In vitro* cytotoxicity evaluation showed that these compounds exhibited high activity against the selected tumor cells. Among them, compound **9** owned the best potential, its IC_{50} values being 5.95 μ mol/L (mononuclear tumor cell line (U937)) and 5.58 μ mol/L (cervical cancer cell (HeLa)). Fluorescence and UV–vis studies showed that compound **9** could bind into the minor groove of DNA.

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1. Introduction

Benzimidazole derivatives are one of the most extensively studied classes of heterocyclic compounds. These compounds have many activities, such as anti-histamine [1], anti-microbial [2], and anti-tumor [3] properties.

Bis-benzimidazole derivatives have been proven to be potent antitumor agents *via* DNA minor groove binding, such as Hoechst 33258 (Scheme 1). Hoechst 33258 [3], a fluorescent compound with a head-to-tail bis-benzimidazole structure, was initially found to be active against L1210 murine leukemia. During phase I clinical trials in humans, positive responses were observed in pancreatic cancer. However, a subsequent phase II clinical trial failed to show any objective responses [4].

Due to the high DNA minor groove binding ability of Hoechst 33258, several groups have synthesized bis-benzimidazole derivatives derived from Hoechst 33258 [5–8]. To the best of our knowledge, most derivatives of Hoechst 33258 are planar molecules, and there is little literature related to adding a linker between two benzimidazoles. Molecular modeling studies (shown in Fig. 1) suggest that the novel benzimidazole (**9**) could effectively bind into the DNA minor groove. Therefore we combined two benzimidazole derivatives into one molecule with an oxygen atom. The interaction

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of compound **9** with CT (calf thymus)-DNA has been investigated using UV–vis, fluorescence spectroscopy. These results showed that compound **9** interacted with the DNA by binding into the minor groove. All of these compounds were screened for anti-tumor activity *in vitro*. Among them, compound **9** had good activity against three tumor cell lines (HeLa, HL60 and U937).

2. Experimental

2.1. Molecular modeling

An AutoDock 3.05 was used to perform theoretical docking calculations [9]. The molecular structure of the A-tract DNA dodecamer d(CGCAAATTTGCG)(PDB code: 2DND) was as receptor. The graphical front-end, AutoDock Tools, was used to add polar hydrogens and partial charges for proteins using the Kollman United Atom charges. Atomic solvation parameters were assigned using the add solubility function in the AutoDock package. Affinity grid fields were generated using the auxiliary program Auto-Grid3.0. The Lamarckian genetic algorithm (LGA) was used to find the appropriate binding positions, orientations, and conformations of the ligands. The optimized AutoDocking parameters are as follows: The maximum number of energy evaluations was increased to 25,000,000 per run; the iterations of Solis & Wets local search was 3000; the number of individuals in the population was 300. All other parameters were maintained as default. Cluster analysis with AutoDock results was performed to determine if different binding sites have been produced from multiple runs.

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Scheme 1. The structure of Hoechst 33258.

2.2. Chemical and instruments

All commercially available reagents and solvents were used without further purification unless otherwise specified. Solvents were dried and re-distilled prior to use according to standard methods. Melting points were determined on a Büchi Melting Point B-540 apparatus (Büchi Labortechnik, Flawil, Switzerland) and are uncorrected. ¹H NMR spectra were measured in DMSO- d_6 on a Bruker ARX 300 spectrometer (Bruker, Rheinstetten, Germany). Chemical shifts are reported in parts per million (ppm) using tetramethylsilane (TMS) as the internal standard if not specifically mentioned (J in Hz). Mass spectra were measured on a Waters Micromass Quattro Micro API mass spectrometer (Waters Corporation, Milford, United States). The UV-vis spectral measurements were recorded on a Cary Varian double beam spectrophotometer (Cary BIO 100, Australia). The sample cuvette used was a pair quartz cells of 1.00 cm path length. All scanning parameters were optimized to obtain the best spectra and in general the parameters were scanned in a wavelength range of 230-300 nm with a step of 0.5 and all measurements were carried out at room temperature. Fluorescence measurements were performed using a Spectrofluorimeter model FS920 of Edinburgh Instruments, U.K. equipped with a xenon arc lamp. The temperature of the sample holder was regulated with a peltier cooled thermostat. Quartz cuvettes of 3 mL capacity and path length 1 cm were used for all measurements.

2.3. General procedure for the synthesis of substituted compounds 8– 15

The key intermediate compounds **3–6** were prepared according to described procedures using 4,4'-diaminodiphenyl ether as a starting material [10]. To a stirred acetone (50 mL) solution of 4,4'-diaminodiphenyl ether (**2**) (5.0 g, 19.6 mmol) at 0 °C, acetic anhydride (10.0 mL, 105.8 mmol) was added dropwise and reacted



Fig. 1. Close-up view of compound 9 binding in the minor groove.

for 3 h. The reaction was monitored by TLC. To stop the reaction, triethylamine (25 mL, 179.4 mmol) was added dropwise, neutralizing the solution and producing a white solid, which was filtrated, washed with acetone, and dried to obtain the compound 3. Then, obtained compound 3 (6.2 g, 16.6 mmol) was dissolved with acetic acid (50 mL) and stirred at 0 °C for 10 min. Fuming nitric acid (8 mL, 171.7 mmol) was added dropwise to the above solution over 2 h. After that, the ice-bath was removed and the reaction mixture was further stirred at room temperature for 2 h. The mixture was poured into ice (100 mL) and the resulting yellow solid (4) was filtered, washed with water, dried in vacuum, dissolved in a mixed water (10 mL) and ethanol (30 mL) solution of potassium hydroxide (7.3 g, 106.7 mmol), and then refluxed for 4 h. After stopped the reaction, the above solution was poured into ice (100 mL). The resulting red solid was filtrated, washed with water, and dried to give 5.2 g (17.9 mmol) of compound **5**. Then, it was dissolved in methanol (50 mL) with Pd-C (0.3 g, 10%) in it, enabling hydrogen gas passing through continuously at a flow rate of 10 mL/min for 3 h. After that, it was filtrated to collect the filtrate and evaporated under vacuum to give 3.7 g of compound 6. Then, compound 6 with potassium ethylxanthate gave 2,2'-dithiol-5,5'-bis-1H,1'H-benzimidazole ether (7), which reacted with various substituted chloromethylpyridine analogs in ethanol at reflux to synthesize the desired compounds 8-15 [11].

2.4. Biological assays

The anti-proliferational effects of the compounds on tumor cells were tested by the same methods. Tumor cells in RPMI1640 medium with 10% fetal bovine serum were plated in 96-well microtiter plates (4.0×10^4 cells per well) and allowed to adhere at 37 °C with 5% CO₂ for 4 h. The test compound was then added, and the cells were incubated at 37 °C with 5% CO₂ for 72 h. The cell viability was assessed using a standard MTT assay [12].

3. Results and discussion

Molecular modeling studies (shown in Fig. 1) suggest that the novel symmetric head-to-head benzimidazole (**9**) could effectively bind into the DNA minor groove. The predicted binding free energy was -14.35 kcal/mol. From the modeling, it was shown that the compound should adopt a concave shape, exactly fitting the convex minor groove, allowing for deeper penetration into the DNA minor groove.

The designed compounds were successfully synthesized as described in Scheme 2. Protection of the amino group of commercially available 4,4'-diaminodiphenyl ether with acetic anhydride at 0 °C gave 4,4'-diacetamido-diphenyl (**3**). Nitration of compound **3** with nitric acid in acetic acid formed compound **4**. Treatment of compound **4** with a solution of potassium hydroxide in water and ethanol gave 3,3'-dinitro-4,4'-diaminodiphenyl ether (**5**), followed by reduction of the nitro group to afford 3,3',4,4'-tetraaminodiphenyl ether (**6**). Treatment of compound **6** with Potassium ethylxanthate gave 2,2'-dithiol-5,5'-bis-1*H*,1'*H*-benz-imidazole ether (**7**), which reacted with sodium hydroxide and various substituted chloromethylpyridine analogs in ethanol at reflux to synthesize the desired compounds **8–15**.

In vitro antitumor activities of all the above final products were screened against three selected tumor cell lines. The biological assay results are summarized in Table 1. It was found that all compounds showed noticeable antitumor activities. Worthy of noting, compounds **8–10** without substituents on the pyridine ring showed more potent antitumor activities than compounds **11–15**, which contain electron-withdrawing halogen substituents (Cl, F or Br) or electron-donating substituents (hydroxyl or methoxyl) on the pyridine ring. Compounds **8–10** showed low cytotoxicity at a concentration of 15 μmol/L, while compounds **11–15** were less potent with IC₅₀



Scheme 2. Reagents and conditions: (a) (CH₃CO)₂O/NEt₃, acetone, 0 °C, 2 h, 98%; (b) conc. HNO₃, acetic acid, 0–50 °C, 5 h, 98%; (c) 42% KOH aq, MeOH, reflux, 2 h, 97%; (d) NH₂NH₂ H₂O, MeOH, r.t., 6 h, 89%; (e) KOH, CS₂, ethanol, reflux, r.t., 6 h, 82%; (f) various hy-CH₂Cl (heterocyclic), NaOH, MeOH, reflux, 3 h, 54–93%.





Compound	Substitute	IC ₅₀ (µmol/L)			
	R	HL60	HeLa	U937	
8	Pyrid-2-yl	12.17	13.83	9.55	
9	Pyrid-4-yl	9.41	5.95	5.58	
10	Pyrid-3-yl	10.36	14.75	14.13	
11	3,5-Dimethyl-4-methoxypyrid-2-yl	>50	30.56	>50	
12	3,4-Methoxypyrid-2-yl	>50	33.64	29.58	
13	3-Methyl, 4-Trifluoroethoxypyrid-2-yl	41.23	>50	>50	
14	3-Methoxyl, 4-chloropyrid-2-yl	29.58	>50	>50	
15	6-Chloropyrid-2-yl	>50	18.77	22.19	
H. 33258		15.62	24.56	31.36	
5-FU		18.23	16.59	13.80	
Paclitaxel		0.004	0.007	0.005	

^a The experiments were performed twice and the average values were obtained from two independent experiments.

values more than 20 μ mol/L. Among them, compound **9** was most potent with IC₅₀ values of 5.95 μ mol/L for the U937 tumor cell line and 5.58 μ mol/L for the HeLa tumor cell line.

UV-vis spectroscopy on CT-DNA in the presence of the small molecules provided useful information related to the nature of the interaction between the two. Fig. 2a shows the absorbance of CT-DNA at 257 nm progressively increasing when the concentration of compound **9** solution was increased from 0 to 5 mmol/L. Not only was there an increase in the absorbance of CT-DNA upon the addition of compound **9**, the appearance of a distinct blue shift upon the formation of the DNA-compound **9** complex in the 257 nm region was also observed. These results indeed indicate that compound **9** may bind in the minor groove of CT-DNA.



Fig. 2. (a) The absorption spectra of calf thymus DNA (1, 7.8×10^{-5} mol/L) in Tris–HCl buffer upon addition of compound **9** (2–5, $1-4 \times 10^{-6}$ mol/L, respectively); (b) fluorescence emission spectra (excited at 520 nm) of EB, EB–DNA complexes in the absence (1) and presence (2–5) of increasing concentrations of the compound **9** (2 mmol/L, 1 L per scan).

Table 2

DNA-binding abilities of compounds 9 and Hoechst 33258 determined by ultrafiltration assay using calf thymus DNA.

Compound	Abs. DNA (–) ^a	Abs. DNA (+) ^a	DNA-binding ability ^b	Scatchard analyses		
				Binding constant (K_a) (×10 ³ L/mol)	Binding sites (n) (per bp)	Correlation coefficient (R)
Hoechst 33258 9	0.468 0.503	0.141 0.172	69.9 65.8	7.3 6.5	0.4 0.4	0.78 0.79

 $^a\,$ The UV-absorption was measured at $\lambda_{max}\,341\,nm$ after 5 dilution with 1.0 buffer.

^b DNA binding ability (%)= $(1 - Abs_{DNA+}/Abs_{DNA-}) \times 100$.

Fluorescence quenching measurements are effective to monitor the binding nature of the small molecules to DNA. The molecular fluorophore EB (ethidium bromide) has a conjugate planar structure, and its fluorescence intensity is very weak, but it emits intense fluorescence at about 600 nm in the presence of DNA due to its strong intercalation between the adjacent DNA base pairs. Many DNA minor groove agents could quench the intense fluorescence [13]. Similar quenching was observed in compound **9** (Fig. 2b). Therefore, it is concluded that compound **9** could bind into the minor groove of DNA.

The DNA-binding ability of compound **9** was also evaluated by the ultrafiltration method (Table 2) [14,15]. It can be seen that the DNA-binding ability for compound **9** was 65.8 and 69.9 for Hoechst 33258. The binding constant (K_a) for compound **9** was 6.5×10^3 L/mol for Hoechst 33258. The similar DNA-binding ability and similar binding constant (K_a) indicate that compound **9** may bind in the minor groove of CT-DNA.

4. Conclusion

In summary, we have successfully designed and synthesized a new series of bis-benzimidazole derivatives based upon molecular modeling experiments of docking within the minor grooves of DNA. These compounds exhibited desirable anti-tumor activity and have better DNA minor groove binding ability. Such compounds are of interest in the context of the future development of novel anti-tumor agents.

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- [11] Analytical data for compounds 7-15:; Compound 7: yield: 54.8%, mp 211.6–213.8 °C; ¹H NMR (300 MHz, DMSO- d_6): δ 6.84 (d, 1H, J = 8.4, Bz-7-H), 7.30 (d, 1H, J = 1.8), 7.52 (dd, 1H, J = 8.4, 1.8, Bz-6-H), 12.61 (br, s, 2H), 13.56 (br, s, 2H); ¹³C NMR (100 MHz, DMS0-d₆); δ 105.6, 112.6, 115.2, 133.8, 138.1, 151.7, 168.0; ESI-HRMS: *m*/*z* 314.0288. (Calcd. for C26H20N6OS2: 314.0296). Compound 8: yield (last step): 92.6%, mp 120.3–122.2 °C; ¹H NMR (300 MHz, DMSO- d_6): δ 4.65 (s, 4H), 6.80–7.20 (m, 4H), 7.27 (m, 2H), 7.36 (d, 2H, J = 7.8 Hz), 7.51 (m, 2H), 7.73 (d, 2H, J = 7.8), 8.51 (m, 2H), 12.65 (br s, 2H); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 41.2, 106.7, 114.2, 115.1, 124.5, 125.3, 134.2, 136.7, 139.4, 148.6, 149.3, 151.9, 159.6; ESI-HRMS: m/z 496.1152. (Calcd. for $C_{c6}H_{20}N_6OS_2$: 496.1140). Compound **9**: yield (last step): 88.7%, mp 118.9–122.2 °C; ¹H NMR (300 MHz, DMSO-*d*₆): δ 4.55(s, 4H), 6.85 (d, 2H, *J* = 8.6 Hz), 7.00 (s, 2H), 7.33 (s, 2H), 7.44 (d, 2H, *J* = 8.6 Hz), 7.86 (d, 2H, *J* = 7.7 Hz), 8.47 (d, 2H, *J* = 7.7 Hz), 8.54 (s, 2H), 12.59 (br s, 2H); ¹³C NMR (100 MHz, DMSO- d_6): δ 39.6, 106.7, 114.2, 115.1, 125.3, 134.2, 139.4, 146.7, 148.4, 148.6, 151.9; ESI-HRMS: m/z 496.1146. (Calcd. for $C_{26}H_{20}N_6OS_2$: 496.1140). Compound 10: yield (last step): 89.3%, mp 134.1–134.9 °C; $^1\mathrm{H}$ NMR (300 MHz, DMSO- d_6): δ 4.53 (s, 4H), 6.83 (dd, 2H, J = 8.7, 2.1 Hz), 6.98 (s, 2H), 7.43 (d, 4H, J = 8.7 Hz), 7.44 (s, 2H), 8.48 (d, 4H, J = 2.1 Hz), 12.60 (br s, 2H); ¹³C NMR (100 MHz, DMSO- d_6): δ 38.3, 106.7, 114.2, 115.1, 125.9, 133.4, 134.2, 134.8, 139.4, 148.6, 151.9, 152.6; ESI-HRMS: m/z 496.1149. (Calcd. for C₂₆H₂₀N₆OS₂: 496.1140). Compound **11**: yield (last step): 90.7%, mp 94.9–96.1 °C; ¹H NMR (300 MHz, DMSO- d_6): δ 3.79 (s, 12H), 3.88 (s, 6H), 4.64 (s, 4H), 6.80–7.20 (m, 4H), 7.07 (d, 2H, J = 5.7 Hz), 8.15 (d, 2H, J = 5.7 Hz), 12.60 (br s, 2H); ¹³C NMR (100 MHz, DMSO- d_6): δ 19.5, 20.6, 42.5, 57.3, 106.7, 114.2, 115.1, 118.4, 132.0, 139.4, 134.2, 143.1, 148.2, 148.6, 150.8, 151.9; ESI-HRMS: m/z 612.1981. (Calcd. for C32H32N6O3S2: 612.1977). Compound 12: yield (last step): 90.7%, mp 94.9-96.1 °C; ¹H NMR (300 MHz, DMSOd₆): δ 3.79 (s, 6H), 3.88 (s, 6H), 4.64 (s, 4H), 6.80-7.20 (m, 4H), 7.07 (d, 2H, J = 5.7 Hz), 7.42 (m, 2H), 8.15 (d, 2H, J = 5.7 Hz), 12.60 (br s, 2H); ¹³C NMR (100 MHz, DMSO-d₆): δ 35.1, 56.2, 56.9, 106.2, 106.8, 114.2, 115.1, 134.2, 142.7, 139.4, 142.6, 148.2, 148.6, 151.9, 157.9; ESI-HRMS: m/z 616.1567. (Calcd. for C30H28N6O5S2: 616.1563). Compound 13: yield (last step): 87.2%, mp 123.7-126.5 °C; ¹H NMR (300 MHz, DMSO-d₆): δ 2.25 (s, 6H), 4.71 (s, 4H), 4.91 (dd, 4H, J = 8.7, 5.4 Hz), 6.80–7.00 (m, 4H), 7.09 (d, 2H, J = 5.4 Hz), 7.45 (s, 2H), 8.31 (d, 2H, J = 8.7 Hz), 12.60 (br s, 2H); 13 C NMR (100 MHz, DMSO- d_6): δ 16.7, 39.0, 83.9, 105.8, 106.7, 112.3, 114.2, 115.1, 124.6, 134.2, 139.4, 148.9, 148.6, 151.9, 161.2, 168.7; ESI-HRMS: m/z 720.1428. (Calcd. for C₃₂H₂₆F₆N₆O₃S₂: 720.1412). Compound **14**: yield (last step): 76.2%, mp 121.8−124.1 °C; ¹H NMR (300 MHz, DMSO-*d*₆): δ 3.96 (6H, s), 4.79 (s, 4H), 6.88 (dd, 2H, *J* = 8.6, 2.1 Hz), 7.04 (s, 2H), 7.47 (d, 2H, *J* = 8.6 Hz), 7.57 (d, 2H, *J* = 2.1 Hz), 8.27 (m, 2H), 12.61 (br s, 2H); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 35.6, 56.3, 106.7, 114.2, 115.1, 122.8, 124.0, 134.2, 139.4, 143.9, 148.6, 149.2, 151.9, 156.2; ESI-HRMS: *m*/*z* 624.0560. (Calcd. for C₂₈H₂₂Cl₂N₆O₃S₂: 624.0572). Compound 15: yield (last step): 53.7%, mp 111.2-113.1 °C; ¹H NMR (300 MHz, DMSO-d₆): δ 4.54 (s, 2H), 6.80-7.30 (m, 2H), 7.43 (m, 1H), 7.46 (d, 1H, J = 8.1 Hz), 7.93 (dd, 1H, J = 8.1, 2.4 Hz), 8.47 (d, 1H, J = 2.4 Hz), 12.61 (br s, 1H); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 39.0, 106.7, 114.2, 115.1, 122.4, 124.1, 134.2, 139.4, 140.6, 148.2, 148.6, 151.9, 160.8; ESI-HRMS: m/z 496.1152. (Calcd. for C26H18Cl2N6OS2: 496.1140).
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