Anticancer Activity of Some Bisbenzimidazoles

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The discovery of DNA topoisomerases has added a new dimension to the study of anticancer drugs. Bisbenzimidazole derivatives are important compounds known as DNA topoisomerase I inhibitors. In the present study, some symmetrical bisbenzimidazole derivatives were synthesized and investigated for their anticancer activity. Anticancer activity screening was applied on HT-29 (colon carcinoma) and MCF-7 (breast carcinoma) cell lines by investigation of cytotoxicity, analysis of DNA synthesis, and DNA fragmentation assays. One of the seven compounds tested showed significant cytotoxicity in both cell lines and caused DNA degradation in the HT-29 cell line.

Key words: Bisbenzimidazole, Anticancer, DNA Topoisomerase

Introduction

The chemotherapeutic action of several anticancer drugs has been related to their capability to inhibit nuclear DNA topoisomerases. Topoisomerases are involved in producing the essential topological and conformational changes in DNA which are critical to many cellular processes such as replication and transcription (Wang, 1985; Liu, 1989; D'Arpa and Liu, 1989). There are two types of topoisomerases, topoisomerase I and topoisomerase II, which have been isolated from mammalian cells. These enzymes can be distinguished due to their function. Mammalian topoisomerase I produces a transient protein-bridged DNA break on one strand, whereas topoisomerase II breaks both DNA strands (Bodley and Liu, 1988; Schneider *et al*., 1990; Chen and Liu, 1994).

Several anticancer drugs in clinical use have been shown to be potent inhibitors of topoisomerase II. Etoposide, teniposide, mitoxantrone, m-AMSA, doxorubicin, and daunomycin are the main examples of this class (Bodley and Liu, 1988; Schneider *et al*., 1990; Chen and Liu, 1994). In comparison to topoisomerase II inhibitors, there are fewer known topoisomerase I inhibitors. Camptothecin is the most extensively studied mammalian topoisomerase I inhibitor. The broad spectrum of potent antineoplastic activity displayed by camptothecin (Gallo *et al*., 1971; Giovanella *et al*., 1991) has resulted in efforts to develop other agents which can be inhibitors of mammalian topoisomerase I. For instance, synthetic bisbenzimidazole derivatives can be considered candidates for this purpose. A bisbenzimidazole derivative, Hoechst 33258, which has been widely studied in relation to its interaction with the DNA double helix, has undergone phase I clinical evaluation as an anticancer agent (Patel *et al*., 1991; Chaudhuri *et al*., 2007). Besides, two analogues, Hoechst 33342 and Hoechst 33377, have recently been reported to have topoisomerase I inhibitory activity (Finlay and Baguley, 1990; Satz *et al*., 2001).

In additon to the Hoechst analogues, a new class of simple symmetrical bisbenzimidazole-based potential DNA minor groove-binding agents was developed by Neidle *et al.* (1997), and the DNA binding capacity of these molecules and their activities as antitumour agents have also been evaluated (Mann *et al*., 2001). In connection with the work of Neidle *et al.* (1997) and after careful literature survey, our goal in the present study was to prepare some different symmetrical bisbenzimidazoles, which have not been investigated for anticancer activity up to date.

Material and Methods

Chemistry

All melting points (M.p.) were determined in open capillaries on a Gallenkamp apparatus

(Weiss-Gallenkamp, Loughborough, UK) and are uncorrected. The purity of the compounds was routinely checked by thin layer chromatography (TLC) using silica gel 60G (Merck, Darmstadt, Germany). Spectroscopic data were recorded with the following instruments: IR, Shimadzu IR-435 spectrophotometer (Tokyo, Japan); ^{1}H NMR, Bruker UltraShield 500 MHz spectrometer (Billerica, MA, USA); LC-MS, Agilent 1100 Series LC/MSD Trap VL & SL (Minnetonka, MN, USA).

*Synthesis of compounds **1**–**8***

The appropriate arylaldehyde (5 mmol) was dissolved in 95% aqueous ethanol (50 mL), and sodium disulfite (5 mmol, 0.95 g) was added. The mixture was refluxed for 1 h, and then the solvent was evaporated. The residue was dissolved in dimethylformamide (DMF) (10 mL) and 3,3'-diaminobenzidine (2.5 mmol, 0.5035 g) was added. The reaction mixture was refluxed at 140 °C for 6 h and then poured into ice water. The precipitated product was filtered, washed with water, dried, and recrystallized from isopropanol.

2,2'-Bis-(2-benzoyloxyphenyl)-1H,1H'-[5,5']-bisbenzimidazole (**1**): Yield 69%. – M.p. 291 °C. – IR (KBr): υ_{max} = 3408 (N-H), 3039 (aromatic C-H), 1746 (ester C=O), 1604–1456 cm^{-1} (C=C and C=N). – ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.16–7.54 (22H, m, benzimidazole, benzoyl and phenyl C_4, C_5 and C_6 protons), 8.60 (2H, d, phenyl C_3 protons), 10.71 (2H, s, N-H protons). – LC-MS: *m/z* = 627.2 $[M+1]^+$.

2,2'-Bis-(anthracen-9-yl)-1H,1H'-[5,5']-bisbenzimidazole (**2**): Yield 82%. – M.p. >350 °C. – IR (KBr): υ_{max} = 3435 (N-H), 3049 (aromatic C-H), 1622–1435 cm^{-1} (C=C and C=N). – ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.36–9.04 (24H, m, benzimidazole and anthracene protons), 11.60 (2H, s, N-H protons). – LC-MS: *m/z* = 587.8 $[M+1]^+$.

2,2'-Bis-(biphenyl-4-yl)-1H,1H'-[5,5']-bisbenzimidazole (**3**): Yield 77%. – M.p. 326 °C. – IR (KBr): υ_{max} = 3433 (N-H), 3030 (aromatic C-H), 1614–1411 cm^{-1} (C=C and C=N). – ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.24–8.16 (24H, m, benzimidazole and phenyl protons), 10.10 (2H, s, N-H protons). – LC-MS: *m/z* = 539.2 $[M+1]^+$.

2,2'-Bis-(3-phenoxyphenyl)-1H,1H'-[5,5']-bisbenzimidazole (**4**): Yield 74%. – M.p. 312 °C. – IR (KBr): υ_{max} = 3483 (N-H), 3061 (aromatic C-H), 1581–1483 cm^{-1} (C=C and C=N). – ^{1}H NMR (500 MHz, $CDCl_3$): δ = 6.94–7.78 (24H, m, benzimidazole and phenyl protons), 10.12 (2H, s, N-H protons). – LC-MS: *m/z* = 571.4 $[M+1]^+$.

2,2'-Bis-(4-phenoxyphenyl)-1H,1H'-[5,5']-bisbenzimidazole (**5**): Yield 78%. – M.p. 234 °C. – IR (KBr): υ_{max} = 3487 (N-H), 3063 (aromatic C-H), 1585–1487 cm^{-1} (C=C and C=N). – ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.08–8.08 (24H, m, benzimidazole and phenyl protons), 10.10 (2H, s, N-H protons). – LC-MS: *m/z* = 571.5 $[M+1]^+$.

2,2'-Bis-(phenanthren-9-yl)-1H,1H'-[5,5']-bisbenzimidazole (**6**): Yield 86%. – M.p. >350 °C. – IR (KBr): υ_{max} = 3417 (N-H), 3059 (aromatic C-H), 1604–1417 cm^{-1} (C=C and C=N). – ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.24–9.38 (24H, m, benzimidazole and phenanthrene protons), 10.40 (2H, s, N-H protons). – LC-MS: *m/z* = 587.4 $[M+1]^+$.

2,2'-Bis-(naphthalen-2-yl)-1H,1H'-[5,5']-bisbenzimidazole (**7**): Yield 81%. – M.p. 341 °C. – IR (KBr): υ_{max} = 3488 (N-H), 3051 (aromatic C-H), 1639–1398 cm^{-1} (C=C and C=N). – ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.28–8.54 (20H, m, benzimidazole and naphthalene protons), 10.50 (2H, s, N-H protons). – LC-MS: *m/z* = 487.4 $[M+1]^+$.

2,2'-Bis-(indol-3-yl)-1H,1H'-[5,5']-bisbenzimidazole (**8**): Yield 75%. – M.p. 276 °C. – IR (KBr): υ_{max} = 3414–3333 (N-H), 3061 (aromatic C-H), 1622–1394 cm^{-1} (C=C and C=N). – ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.24–7.96 (16H, m, benzimidazole and indole protons), 10.32–10.46 (4H, s, N-H protons). – LC-MS: *m/z* = 465.6 $[M+1]^+$.

Cell cultures

MCF-7 (breast carcinoma) cells were maintained in 90% Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA), 1 mM sodium pyruvate (Sigma), 10 μg/mL human insulin (Sigma), and 10% (v/v) fetal bovine serum (FBS) (Gibco, Paisley, UK). HT-29 (colon carcinoma) cells were cultured in 90% McCoy's 5A medium (Sigma) and 10% FBS (Gibco). All media were supplemented with penicillin/streptomycin at 100 units/mL, and cells were incubated at 37 °C in a 5% CO_2/95% air humidified atmosphere.

MTT assay

A tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), was used as a colorimetric substrate for measuring the cytotoxicity (MTT assay). HT-29 and MCF-7 cells were cultured in 96-well plates, and 1.563 to 200 μg/mL bisbenzimidazole derivative or cisplatin was added. The plates were incubated for 24 h at 37 °C in a 5% CO_2/95% air humidified incubator together with an untreated control sample. After incubation, 20 μL MTT dye were added, and the absorbance of the plates was measured with an ELx808-IU Bio-Tek apparatus (Bio-Tek Instruments Inc., Winooski, USA) at 540 nm. Control cell viability was regarded as 100%. Stock solutions of the test compounds were dissolved in dimethyl sulfoxide (DMSO), and further concentrations were prepared in cell culture media. All experiments were repeated three times. For each dose of a compound, four independent wells were used. Percent viability was defined as the relative absorbance of treated versus untreated control cells.

DNA synthesis inhibition assay

Tests for DNA synthesis inhibitory effects of the synthesized compounds were performed in 96-well flat-bottomed microtiter plates using a BrdU colorimetric kit (Roche, Penzberg, Germany). HT-29 and MCF-7 cells were collected from cell cultures by 0.25% trypsin/EDTA solution and counted in a hemocytometer. Suspensions of the cell lines were seeded into 96-well flat-bottomed microtiter plates at a density of 10^3 cells/mL. The tumour cell lines were cultured in the presence of various doses of the test compounds or cisplatin. Microtiter plates were incubated at 37 °C in a 5% CO_2/95% air humidified atmosphere for 24 h. The cells were labelled with 10 μL BrdU solution for 2 h and then fixed. Anti-BrdU-POD (100 μL) was added and incubated for 90 min. Finally, the microtiter plates were washed three times with phosphate buffered saline (PBS), and the cells were incubated with substrate solution until the colour turned blue. Absorbance of the samples was measured with an ELx808-IU Bio-Tek apparatus at 492 nm. As a control solvent, DMSO was added to the cells during the time course. The values of blank wells were substracted from the value of each well of treated and control cells. The absorbance values of background control wells did not exceed 0.1. All experiments were repeated three times. For each dose of the compounds, triplicate wells were used.

DNA fragmentation assay

HT-29 cells (10^6 cells/mL) were harvested into dishes and incubated with various doses of the test compounds for 24 h. After the incubation period, cells were washed in PBS, trypsinized, transferred into tubes, and mixed. Binding buffer (200 μL) was added and the mixture incubated at room temperature for 10 min; then isopropanol (100 μL) was added and the mixture mixed. The mixture was filtered and centrifuged at 8000 x *g*. Washing buffer (500 μL) was added, and the resulting mixture was centrifuged again at the same *g* value. Filter tubes were solubilized with elution buffer and analysed by 1% agarose gel electrophoresis (containing 500 μg/mL of ethidium bromide) at 50 V for 120 min. Approximately 20 μg of DNA was loaded in each well, visualized under UV light, and photographed.

Statistics

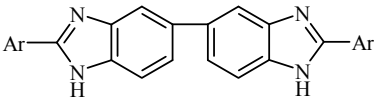
The SPSS for Windows 11.5 computer program was used for statistical analyses. Statistical comparison of the results obtained from controls, groups, and time periods were carried out by the one-way analyses of variance (ANOVA) test, and post hoc analyses of group differences were performed by the Tukey test. Results were expressed as mean ± SD.

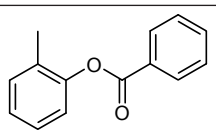
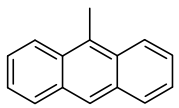
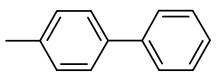
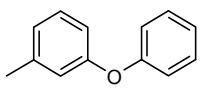
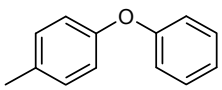
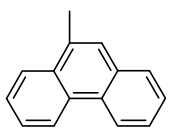
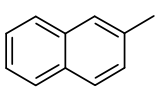
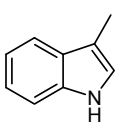
Results and Discussion

In the present work, 2,2-bis-aryl-1*H*,1*H*'-[5,5']-bisbenzimidazole derivatives **1**–**8** were synthesized by reacting the sodium disulfide adduct of appropriate aldehyde derivatives with 3,3'-diaminobenzidine. The chemical structures of compounds **1**–**8** were confirmed by IR, ^{1}H NMR, and mass spectral data. Chemical formulas of the compounds are presented in Table I.

In the IR spectra, some significant stretching bands due to N-H, aromatic C-H, and C=N and C=C vibrations were determined at 3488–3408 cm^{-1}, 3030–3063 cm^{-1}, and 1639–1394 cm^{-1}, respectively. In the ^{1}H NMR spectra, the signals of N-H protons were observed at 10.10–11.60 ppm and clearly separated from the other peaks. The peaks belonging to aromatic protons were recorded at a wide area of 6.94–9.04 ppm. The mass spectra of the compounds showed $[M+1]^+$ peaks, in agreement with their molecular formula.

Table I. Molecular formulas and IC_{50} values of compounds **1**–**8** against the HT-29 and MCF-7 cell lines.



Compound	Ar	HT-29 IC_{50} [μg/mL]	MCF-7 IC_{50} [μg/mL]
1		40.4*	150.6
2		15.7**	186.2
3		135.2	80.4*
4		26.5**	80.8*
5		125.3	180.7
6		133.9	100.4
7		9.5**	4.1**
8		8.5**	190.9
Cisplatin	-	1.7	2.6

The compounds displaying moderate (*) and significant (**) activity against cancer cell lines were selected for DNA synthesis inhibition and assays for the detection of apoptotic DNA fragmentation.

Anticancer activity screening of compounds **1**–**8** against HT-29 and MCF-7 cell lines was performed in three steps. In the first step, cytotoxicity of the compounds was determined by the MTT method. Afterwards, DNA synthesis inhibition was analysed for the compounds possessing significant cytotoxic activity. Finally, DNA degradation caused by compounds was monitored in a UV gel visualization system.

In the MTT test, HT-29 and MCF-7 cell lines were incubated with compounds **1**–**8** at various concentrations (1.563, 3.125, 6.25, 12.5, 25, 50, 100, and 200 μg/mL). After 24 h incubation, cytotoxic effects of the compounds were examined and IC_{50} values calculated. The anticancer agent cisplatin was used as a positive control. As seen in Table I, compound **7** was the most substantial derivative in the series, since it exhibited significant cytotoxicity against both cancer cell lines. Moreover, the cytotoxic effect of this compound is very close to that of cisplatin (IC_{50} = 2.6 μg/mL) against the MCF-7 cell line. Besides, compounds **1**, **2**, **4**, and **8** were found to be active against the HT-29 cells with IC_{50} values of 40.4 μg/mL, 15.7 μg/mL, 26.5 μg/mL, and 8.5 μg/mL, respectively. Compounds **3** and **4** showed moderate cytotoxic activity against the MCF-7 cell line. The MTT test results revealed that, in general, MCF-7 cells were more resistant against the tested compounds, while HT-29 cells showed more sensitivity. Due to their notable cytotoxicity, compounds **1**–**4**, **7**, and **8** were chosen for the DNA synthesis inhibition assay.

In the DNA synthesis inhibition assay, for a 24-h time period, HT-29 and MCF-7 cells were incubated with the compounds at three different doses (IC_{50}/4, IC_{50}/2, and IC_{50}). Cisplatin was used as a positive control. As seen in Figs. 1 and 2, the test compounds showed dose-dependent inhibitory activity on DNA synthesis in both cell lines. Although, the inhibitory activity observed at the lowest dose of the compounds was very poor, all of the compounds showed inhibitory activity similar to that of the reference at the highest dose (IC_{50}). However, this finding was of little relevance for compounds **1**, **2**, **3**, and **4** because their doses had to be 5- to 25-fold higher than that of cisplatin to exhibit similar activity. On the other hand, DNA synthesis inhibitory activities of compound **7** against both cell lines and compound **8** against the HT-29 cell line were more notable, since these compounds showed similar DNA synthesis inhi-

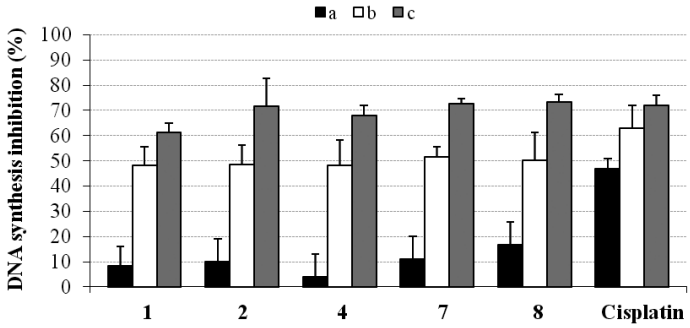


Fig. 1. Inhibitory effects of compounds **1**, **2**, **4**, **7**, and **8** on DNA synthesis of HT-29 cells. Mean percentage of absorbance of the untreated (assessed in the presence of DMSO used as a solvent and assumed as 0%) and treated cells (test compounds with three different concentrations, a, b, c) and cisplatin are given. **1**: a, 10 μg; b, 20 μg; c, 40 μg. **2**: a, 4 μg; b, 8 μg; c, 16 μg. **4**: a, 6.5 μg; b, 13 μg; c, 26 μg. **7**: a, 2.375 μg; b, 4.75 μg; c, 9.5 μg. **8**: a, 2.125 μg; b, 4.25 μg; c, 8.5 μg. Cisplatin: a, 0.64 μg; b, 1.7 μg; c, 3.2 μg. Data represent means for three independent experiments ± SD of nine independent wells. $p < 0.05$.

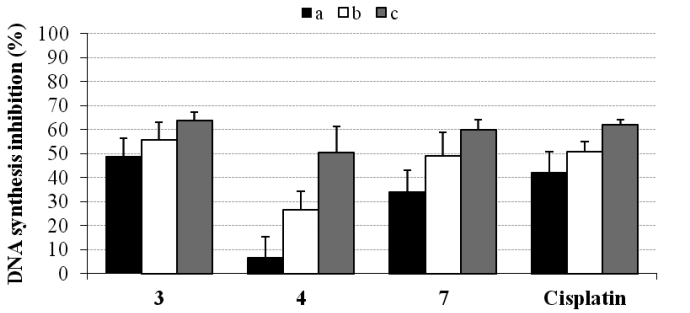


Fig. 2. Inhibitory effects of compounds **3**, **4**, and **7** on DNA synthesis of MCF-7 cells. Mean percentage of absorbance of the untreated (assessed in the presence of DMSO used as a solvent and assumed as 0%) and treated cells (test compounds in three different concentrations, a, b, c) and cisplatin are given. **3**: a, 20 μg; b, 40 μg; c, 80 μg. **4**: a, 20 μg; b, 40 μg; c, 80 μg. **7**: a, 1 μg; b, 2 μg; c, 4 μg. Cisplatin: a, 0.64 μg; b, 1.7 μg; c, 3.2 μg. Data represent means for three independent experiments ± SD of nine independent wells. $p < 0.05$.

bition compared to the reference at lower doses compared to the other compounds.

The DNA fragmentation assay was carried out for the most cytotoxic compounds **7** and **8** against the HT-29 cell line, because this cell line was more sensitive to the compounds. HT-29 cells were incubated with the compounds at two different concentrations (IC_{50} and $IC_{50}/2$). After a 24-h incubation period, DNA was isolated from the cells, electrophoresed along with the apoptotic DNA ladder kit, and monitored in a UV gel visualization system. As seen in Fig. 3, both doses of compounds **7** and 8 caused DNA degradation, but not laddering, which is a marker of apoptotic DNA (lanes 1, 2, 3, and 4). This result suggests that the test compounds damage DNA which causes the anticancer action.

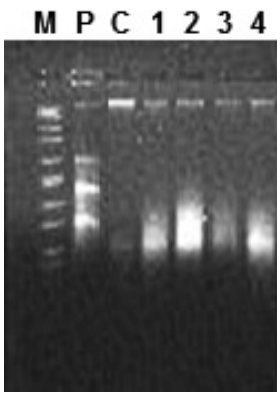


Fig. 3. Effects of compounds **7** and **8** on the genomic DNA of HT-29 cells. M, DNA marker (10000–50 bp); P, positive control (DNA of HT-29 cell line treated with 4 μg/mL of the reference drug camptothecin); C, control (DNA of HT-29 cell line); 1, DNA of HT-29 cell line treated with 4.75 μg/mL of compound **7**; 2, DNA of HT-29 cell line treated with 9.5 μg/mL of compound **7**; 3, DNA of HT-29 cell line treated with 4.25 μg/mL of compound **8**; 4, DNA of HT-29 cell line treated with 8.5 μg/mL of compound **8**.

In conclusion, the preliminary *in vitro* anticancer investigation of some novel bisbenzimidazole derivatives has indicated the anticancer potency of compounds **7** and **8**. Compound **7** has more chemotherapeutic value than compound **8**, because, the 2-naphthyl-substituted compound **7** showed an attractive cytotoxicity against both cell lines tested. The 3-indolyl-substituted compound **8** had a significant cytotoxic activity only against HT-29 cell line. The results reveal that the anticancer activity of compounds **7** and **8** is related to their cytotoxic and DNA degradation effects, respectively, on the HT-29 cell line.

Acknowledgement

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