European Journal of Medicinal Chemistry 46 (2011) 659–669

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and biological activity of novel inhibitors of topoisomerase I: 2-Aryl-substituted 2-bis-1H-benzimidazoles

Manish Singh [a,b], Vibha Tandon [a,*]

[a] Department of Chemistry, University of Delhi, Delhi 110007, India
[b] Dr. B.R. Ambedkar Center for Biomedical Research, University of Delhi, Delhi 110007, India

ARTICLE INFO

Article history:
Received 26 March 2010
Received in revised form
25 November 2010
Accepted 30 November 2010
Available online 8 December 2010

Keywords:
Bisbenzimidazole
MTT
Hoechst 33342
Antitumor activity
Topoisomerase I
Calf Thymus DNA

ABSTRACT

Inhibitors of topoisomerase I constitute a novel family of antitumor agents. The class of benzimidazole derivatives contains compounds possessing affinity to DNA. For example, fluorescent stains **Hoechst 33342** and **Hoechst 33258** interact with DNA as ligand and produce nonspecific inhibition of the catalytic activity of many enzymes involved in DNA synthesis, including DNA topoisomerase and DNA helicase. Several 2-aryl-5-substituted-2,5-bisbenzimidazole derivatives were synthesized and ability of these derivatives to induce DNA cleavage in the presence of topoisomerase I was evaluated in vitro. These analogs were also assayed for their cytotoxicity against U87, MCF7 and HeLa human tumor cells. All the four compounds showed a potent growth inhibitory effect on all the cell lines, with IC_{50} in the μM range.

1. Introduction

Studies on the noncovalent interactions of small molecules with the minor groove of DNA continue to be a fruitful area for the discovery of potential new therapeutic agents. The main representatives of this class, which reached the clinic, are the antitumor agents derived from CC-1065, that is, adozelesin, carzelesin and bizelesin, and the distamycin A derivative tallimustine. These 'classical' minor groove binding agents (MGBs) have been shown to be highly DNA sequence-specific [1,2] and to exert their cytotoxic effect through the ability to directly alkylate DNA mainly at the N(3) position of adenines exposed in (AT) rich sequences in the DNA minor groove [3–7]. While certain DNA minor groove binding agents, berenil, netropsin and various symmetric and asymmetric bisbenzimidazoles can block DNA topoisomerase and helicase activity by binding to duplex at specific base sequences [8,9].

DNA topoisomerases are ubiquitous enzymes that catalyze essential enzymes to solve the topological problems accompanying key nuclear processes such as DNA replication, transcription, repair and chromatin assembly by introducing temporary single or double strand breaks in the DNA [10–13]. In addition, these enzymes fine-tune the steady-state level of DNA supercoiling to facilitate protein interactions with DNA and to prevent excessive supercoiling. There are two fundamental types of topoisomerases, which differ in both mechanism and cellular function [10]. Type I DNA topoisomerases are classified into two subfamilies: type IA and type IB. The enzymes of type IA subfamily, including bacterial DNA topoisomerase I and II, eukaryotic DNA topoisomerase III and reverse gyrase [14,15] form a tyrosyl linkage with a 5′-phosphate group of the DNA strands generated due to the enzyme action [11], whereas the enzymes of type IB subfamily, including eukaryotic and vaccinia virus DNA topoisomerases I [16] and topoisomerase V, establish the tyrosyl bond with the 3′-phosphate group [11]. Type IA topoisomerases relax only negative supercoiled DNA with Mg^{2+} requirement, whereas type IB topoisomerases relax both negative and positive supercoiled DNA even in the absence of metallic cofactors, although Mg^{2+} and Ca^{2+} stimulate the relaxation activity [17,18].

Hoechst 33258, also known as Pibenzimol, which has two benzimidazole groups linked in a head-to-tail manner [19]. This compound was reported to have moderate in vitro activity on L1210 leukemia on repeated dose schedule but was not active against P388 using the single-dose protocol [20] which was followed by several phase I trials in humans [21]. The first trial was limited by pancreatic toxicity; although in a second trial some responses were seen in pancreatic cancer, a subsequent phase II trial did not show any objective responses [22] and no further trial of this agent have

* Corresponding author.
E-mail addresses: vibhadelhi@hotmail.com, vtandon@acbr.du.ac.in (V. Tandon).

0223-5234/$ – see front matter © 2010 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2010.11.046

been reported. Analogs of **Hoechst 33258**, bearing alkylation of the terminal phenoxy moiety enhanced cytotoxic potency with respect to parent compound **Hoechst 33258**. About the phenolic ether of **Hoechst 33258**, such as **Hoechst 33342** ($R = OC_2H_5$) or **Hoechst 33377** ($R = OC_6H_5$), it has been suggested that these agents exhibit greater cytotoxicity due to their superior ability to traverse the cell membrane and reach its DNA target [23]. In fact, due to the ability of these compounds in traversing both the cytoplasmic and nuclear membranes and accumulating in the nucleus, they are capable of inhibiting the binding of regulatory proteins. X-ray and NMR studies of **Hoechst 33258** bound to DNA as a 1:1 complex [24–26] indicate how molecule accomplishes recognition of DNA: similar to netropsin and distamycin, specific H-bonds are formed between the benzimidazole NHs and adjacent adenine N(3)- and thymine O (2)-atom on the floor of the minor groove. The binding preference for AT base pairs is a result of the closer contact of the aromatic H-atoms of Hoechst molecule and floor of the minor groove, thus precluding the binding of the drug at GC base pairs due to the presence of exocyclic N(2)-amino group of guanine. In addition, the binding of Hoechst is stabilized by electrostatic interaction and extensive van der Waals contacts with the walls of the minor groove. Earlier, several analogs of this bisbenzimidazole have been synthesized to further investigate the structure–activity relationships associated with their potency as topoisomerase I inhibitors and the related cytotoxicity [27] (Fig. 1).

Most derivatives of **Hoechst 33258** having the monosubstitution at para or meta position of phenyl ring, which kept whole molecules to planar structures. To our knowledge, no literature related to di-substituted groups, having halogen atoms as substituents, at 2-position of benzimidazole as the derivatives of Hoechst were reported so far. Moreover affinity increase in order for the three DNA: p-OH < m-OCH_3, p-OH < m-OH, p-OCH_3 < bis-m-OH Hoechst derivatives having various substitution (OH, m-OH, p-OCH_3, m-OCH_3, bis-m-OH) on phenyl ring to poly[d(A-T)] [28].

Our interest in biologically active compounds as potential antitumor agents focused our studies on the synthesis of several di and tri substituted bisbenzimidazole, which have electron donating (OCH_3) and electron withdrawing groups (F, Cl) on the phenyl ring and evaluate their potential to induce DNA cleavage in the presence of mammalian topoisomerase I. The results associated with the cytotoxicity observed for these analogs in various tumor cell lines is also being presented in this manuscript.

2. Chemistry

Traditionally, benzimidazoles have most commonly been prepared from the reaction of 1,2-diaminobenzenes with carboxylic acids under harsh dehydrating reaction conditions, utilizing the strong acid such as polyphosphoric acid, hydrochloric acid, boric acid or p-toluenesulfonic acid [29]. However, the use of milder reagents, particularly Lewis acids, [30] inorganic clays [31] or mineral acids [32] has improved both the yield and purity of the reaction [33]. On the other hand, the synthesis of benzimidazoles via the condensation of 1,2-diaminobenzenes with aldehydes requires an oxidative reagent to generate the benzimidazole nucleus. Various oxidative reagents, such as nitrobenzene, benzoquinone, mercuric oxides, lead tetra acetate, iodine, copper(II) acetate and Fe(III) mediated oxidation have been employed for this purpose [34]. However, we determined during our early attempts that such reported procedure for preparation of benzimidazole derivatives was not quite versatile nor compatible with different substituted starting materials and also posed practical difficulties in the form of laborious reaction workup protocols. The dehydrogenating capacity of bisulfite has been demonstrated in the condensation of 1,8 diaminonaphthalene and aldehyde to 2-substituted-1H-pyrimidines [35,36]. Hence, we are utilizing bisulfite as an oxidizing reagent and condensation was carried out in ethanol. Therefore, bisbenzimidazole derivatives **6a**–**d** were easily prepared by using this procedure. A series of 2-aryl-5-cyano-1H-benzimidazole **2a**–**d** were synthesized by coupling of 4-cyano-1,2-phenylenendiamine with the appropriate substituted benzaldehydes (Scheme 1). All benzimidazoles **2a**–**d** were synthesized in yields ranging from 65% to 85%. Reduction of **2a**-**d** with Ni-Al alloy in the presence of formic acid followed by hydrolysis provides an effective method for preparation of the 2-aryl-5-formyl-1H-benzimidazole intermediates **3a**-**d** (Scheme 2) required for the formation of final compounds i.e. 2,5-bi-1H-benzimidazole used in this study. The treatment of 5-chloro-2-nitroaniline with 4-methylpiperazine in the presence of anhydrous potassium carbonate in DMF afforded the required 5-(4-methyl-1-piperazinyl)-2-nitroaniline **4** in nearly 85% yield. The required diamine **5** was obtained by catalytic hydrogenation of compound **4**, using palladized carbon in a Parr reactor at ambient temperature. The preparation of all four 2′-aryl-5-(4-methylpiperazinyl)-2,5′-bi-1H-benzimidazoles **6a**–**d** was accomplished by coupling of 5-(4-methylpiperazinyl)-1,2-phenylenediamine with the appropriate 2-aryl-5-formyl-1H-benzimidazole (Scheme 2).

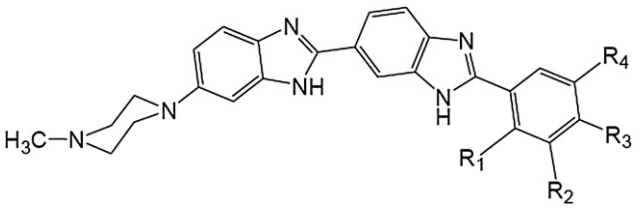
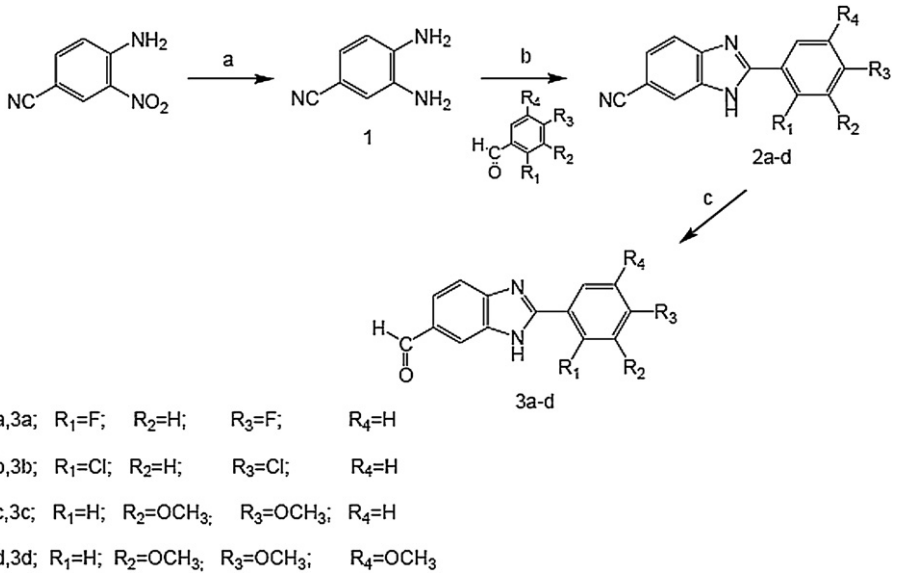


Fig. 1. Chemical structure of bisbenzimidazoles for **Hoechst 33258**, $R_1 = R_2 = R_4 = H$, $R_3 = OH$; **Hoechst 33342**, $R_1 = R_2 = R_4 = H$, $R_3 = OC_2H_5$; **Hoechst 33377**, $R_1 = R_2 = R_4 = H$, $R_3 = OC_6H_5$; **6a**, $R_2 = R_4 = H$, $R_1 = R_3 = F$; **6b**, $R_2 = R_4 = H$, $R_1 = R_3 = Cl$; **6c**, $R_1 = R_4 = H$ $R_2 = R_3 = OCH_3$; and for **6d**, $R_1 = H$, $R_2 = R_3 = R_4 = OCH_3$.

3. Results and discussion

3.1. DNA binding

To understand the physico-chemical properties of the compounds, absorbance measurements were done in 20 mM sodium cacodylate buffer, 100 mM NaCl, pH 7.2. **Hoechst 33342** (hereafter **Hoechst**), **6c** and **6d** showed absorption maxima at 340 nm whereas **6a** and **6b** showed absorption maxima at 334 nm (Table 1). In the present paper we studied the interaction of above four compounds with CT-DNA using absorbance spectroscopy, where changes in absorbance and absorption maxima indicated binding. The absorption maxima for **Hoechst**, **6c** and **6d** showed a bathochromic shift of 10, 9 and 7 nm respectively at $r = 4$, DNA/drug ratio. Compounds **6a** and **6b** showed a bathochromic shift of 12 and 6 nm respectively. **Hoechst**, **6c** and **6d** showed 38%, 28.1% and 27.1% hypochromicity at $r = 4$, DNA/drug ratio, whereas in the case **6a**, 20% hyperchromicity was observed only (Fig. 2). In the case of **6c** we did not observe any significant changes in absorbance. A significant amount of hyperchromicity as well as 12 nm bathochromic shift observed in case of **6a** after binding to CT-DNA can be attributed to the fluorine substitution at phenyl ring resulting in a strong binding between compound and DNA. As our results suggest that **6c**, **6d** and **6c** after binding to CT-DNA observed hypochromic shift whereas only **6a** had shown hyperchromicity. The size of fluorine atom is smallest as it has a small radii, because of which **6a** may not only be behaving as a minor groove binder rather it is able to slip between DNA base pairs and bind to them as an intercalator causing the unwinding of DNA duplex and resulting into the hyperchromicity



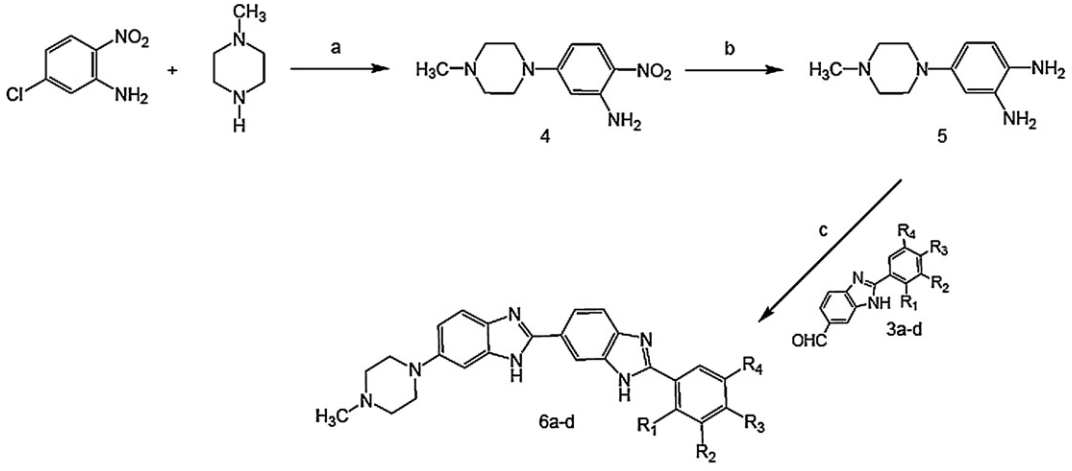
Scheme 1. Reagents, condition and yield: a) H_2, 10% Pd/C, EtOH, rt, 30 min, 95%. b) $Na_2S_2O_5$, MeOH/H_2O, reflux 5 h, 65–85%; c) Ni–Al alloy, formic acid, 95 °C, 5 h, 55–60%.

shift in UV absorption. Hence on the basis of these results we can suggest that **6b**, **6c** and **6d** are binding with CT-DNA in a non cooperative manner similar to **Hoechst** whereas **6a** has a cooperative binding with CT-DNA.

Upon binding to CT-DNA, fluorophore (**6a–d**, **Hoechst**) exhibits a significant fluorescence enhancement, due to the loss of solvent exposure upon snugly binding (van der Waals and H-bonding forces) within the DNA groove (Fig. 3a). Ligands **6a–d** and **Hoechst** showed single emission maxima in presence of CT-DNA. Ligands (**6a**, **6c**, **6d**) showed a blue shift of 13–31 nm, whereas 44 nm blue shift was observed in case of **Hoechst**. The low dissociation constant for **6a** and **6b** (1.86×10^{-6} and 1.98×10^{-6} respectively) as compared to reference ligand **Hoechst** (4.50×10^{-6}) suggested that halogenated bisbenzimidazoles (**6a** and **6b**) showed greater binding affinity as compared to methoxy substituted bisbenzimidazoles (**6b** and **6d**) as well as **Hoechst** (Fig. 3b).

3.2. Cytotoxicity

Chemotherapy is a major therapeutic approach for the both localized and metastasized cancers. The newly synthesized bisbenzimidazole derivatives **6a**, **6b**, **6c** and **6d** were evaluated for their cytotoxicity against human tumor cell lines, which are cervix carcinoma cell line (HeLa), breast carcinoma cell line (MCF7) and brain glioma cell line (U87) in comparison to **Hoechst** and **Camptothecin** as reference compound (Fig. 4a–c). The MTT assay was widely applied to examine in vitro cytotoxicity. Compounds **6a–d** had inhibitory effects on the growth of tumor cells (U87, MCF7 and HeLa)



Scheme 2. Reagents, condition and yield: a) Methylpiperazine, anhydrous K_2CO_3, dry DMF, 110 °C, 24 h 85%. b) H_2, 10% Pd/C, EtOH, rt, 2 h, 95%. c) $Na_2S_2O_5$, EtOH/H_2O, reflux 5 h, 62–68%.

Table 1
UV–visible spectral changes of the bisbenzimidazoles derivatives upon titration with CT-DNA at DNA/drug ratio $r = 4$.

Compound	$\lambda_{ex}/\lambda_{em}$ (nm)	Red shift	Hypochromicity	log *P*
Hoechst 33342	340/496/	10	38	3.95
6a	334/501	12	−20	4.05
6b	334/[a]	6	28	4.85
6c	340/478	9	1	3.48
6d	340/514	7	27	3.36

A negative hypochromicity means a corresponding hyperchromicity.
[a] Single emission value was not observed for **6b**.

in dosage and time-dependent manners except compounds **6c** and **6d** in case of U87 (Fig. 4c). Meanwhile **6a**, **6b** showed higher antiproliferative activity than **6c** and **6d** against all screened cell lines, demonstrating that our strategy of modifying these molecules was successful (Table 2). Earlier it was observed in our laboratory that dimethoxy analog of bisbenzimidazole (**6c**) is non cytotoxic in comparison to Hoechst in **BMG-1** (human glioma) cell lines [37]. The non cytotoxicity may be due to low cellular uptake of these compounds as their log *P* is 3.48 which is less than the **Hoechst** i.e., 3.95. This trend was further confirmed by the synthesis of trimethoxy analog **6d** having log *P* value 3.36. The cellular uptake of **6c** and **6d** is reduced due to less lypophilicity of these compounds. The IC_{50} value could not be determined even at 100 μM concentrations of **6c** and **6d** for U87 cell line (72 h). But in case of MCF7, the IC_{50} was observed at 5.3 and 16.8 μM for **6c** and **6d** respectively. The IC_{50} determined in the case of HeLa was 3.4 and 29 μM for **6c** and **6d** respectively.

Compound **6a** was approximately 14–18 fold more cytotoxic than **6c** and **6d** against U87 cell lines and **6b** was even 3.6-fold more cytotoxic than **6a** and 2.8-fold more potent than **Hoechst**, but nearly 3-fold less cytotoxic than **CPT**. This analog (**6b**) was also active against MCF7 and HeLa cells, but it was almost 2.6-fold and 2-fold less potent with respect to the **Hoechst**. In case of Hela cells **6b** was observed 3-fold more cytotoxic than **CPT**. The IC_{50} values of **6a** and **6c** were 5.5 and 5.3 μM against MCF7 whereas in HeLa, compound **6a** was 2.2-fold (IC_{50}; 1.5 μM) more potent than **6c** (IC_{50}; 3.4 μM) at 72 h. Compound **6d** was less potent in case of all the three above mentioned cell lines. On the basis of above results it can be suggested that **6b** is more toxic in comparison to **6a**, **6c**, **6d** and its toxicity is comparable to **Camptothecin** in HeLa cells and little less in U87 and MCF7 also. Certainly the analog **6b** may serve as important lead in the synthesis of more potent and selective anticancer agents.

3.3. Topoisomerase I inhibition

To investigate the inhibitory activity of these molecules against purified human topo I, the enzyme was purified following the

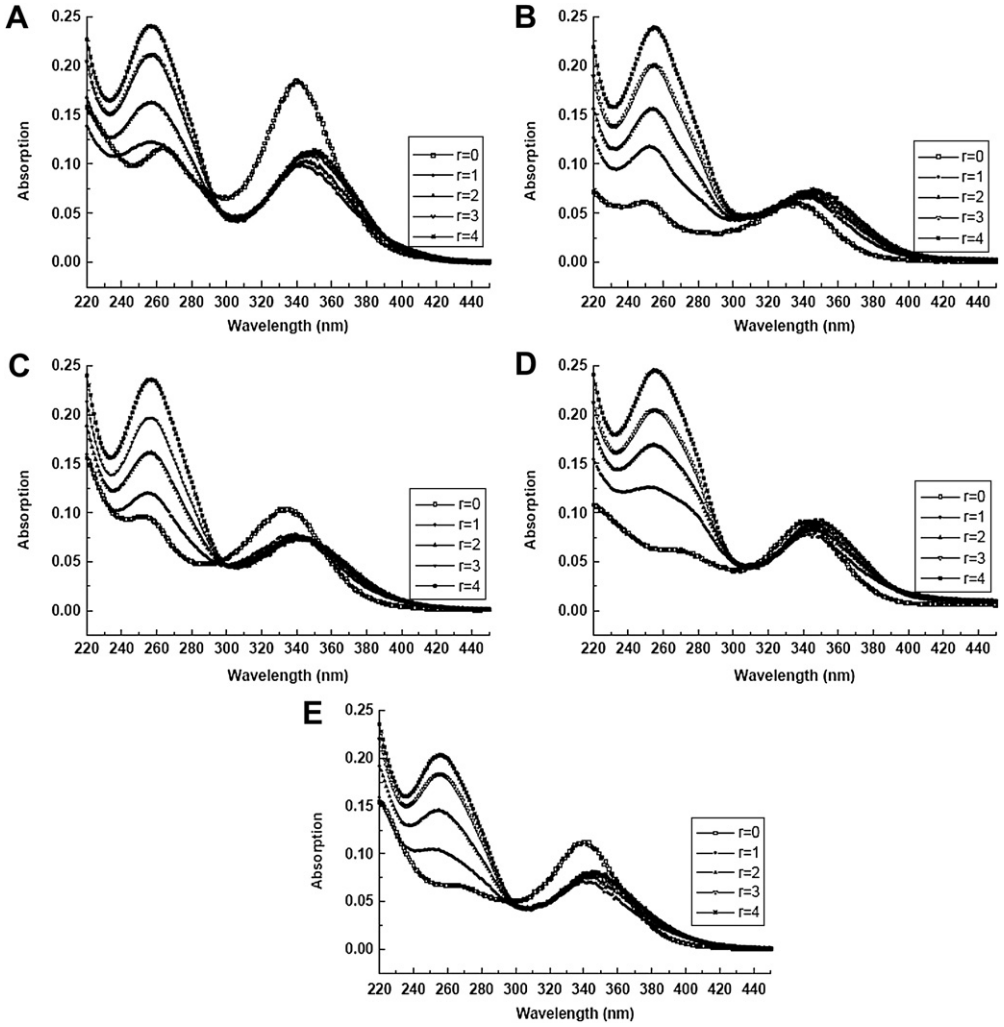


Fig. 2. UV absorption spectra of **Hoechst 33342** (A), **6a** (B), **6b** (C), **6c** (D) and **6d** (E) alone and in the presence of CT-DNA. The compound concentration was 5 μM:free, $r = 0$, $r = 1$, $r = 2$, $r = 3$, $r = 4$.

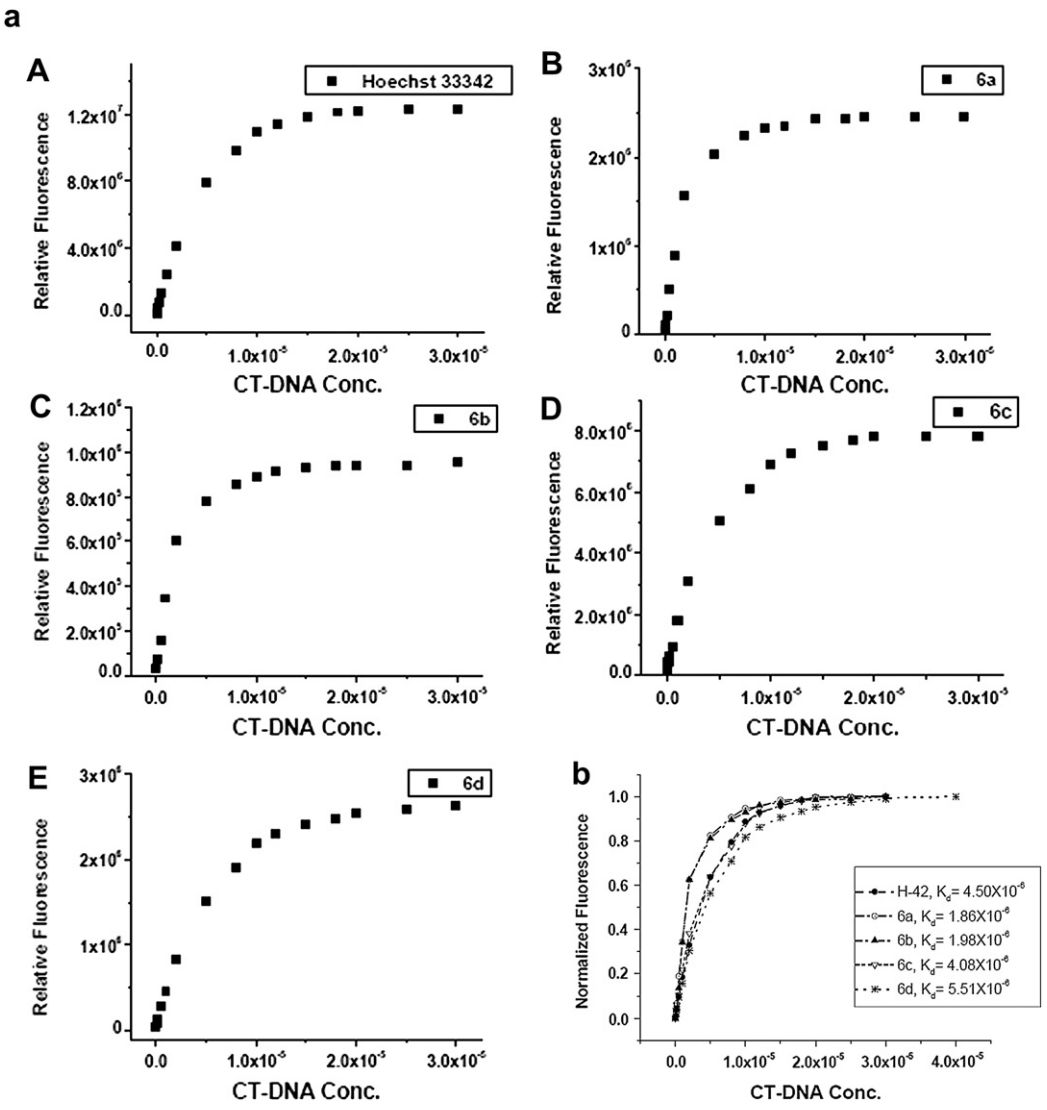


Fig. 3. a. A change in the fluorescence emission of ligand (**6a–d** and **Hoechst 33342**) was observed on addition of CT-DNA at concentrations of 0.1–40 μM. All measurements were made after preincubating ligand with or without DNA at 25 °C for 5 min. b. Plot of Δ_F/Δ_{Fmax} versus increasing concentration of CT-DNA.

procedure given in literature. A relaxation assay was performed by simultaneous incubation of supercoiled plasmid, pBS (SK+) DNA, enzyme and compounds. The inhibitory activity of the compounds was compared to that of standard topo I poison **Camptothecin** (Fig. 5). **Camptothecin** have shown topoisomerase I inhibition below 10 μM concentration. which is evident from reduced topoisomer bands and ~80% recovery of the supercoiled DNA band. The di and trimethoxyphenyl bearing compounds **6c** and **6d** showed less enzyme inhibition. The ~90% topoisomerase activity could be inhibited at ~50 μM and ~75 μM concentration of **6c** and **6d**. Dihalogenated phenyl bearing derivatives **6a** and **6b**, however, showed effective inhibition of the enzyme activity even at ~25 μM concentration. This was evident from the recovery of the supercoiled DNA band with increasing concentration of above compounds. Compounds **6a** and **6b** were found to be more potent topo I inhibitors, as compared to reference compound **Hoechst**.

4. Conclusion

In view of above results we can conclude that introduction of halogen groups at the phenyl ring not only increases the binding affinity of benzimidazole to CT-DNA but in addition to it, fluoro and chloro (**6a** and **6b**) showed significant cytotoxicity to human tumor cell lines in comparison to methoxy substituted analogs of bis-benzimidazole (**6c** and **6d**). These compounds can be further pruned to develop a chemotherapeutic drug against cancer.

5. Experimental protocols

5.1. Chemistry

All the starting materials and reagents were purchased from E. Merck (Germany), S.D. Fine (India), Spectrochem (India), Aldrich and Fluka were used without further purification. Solvents were dried and redistilled prior to use using standard method. Melting points were recorded on Buchi 540 apparatus. The purity of the compounds was confirmed by thin layer chromatography (TLC) using Merck silica gel 60 F_{254} coated alumina plates and detected under UV-light or I_2 vapor. Column chromatography was performed using silica gel mesh size 60–120. Infrared (IR) spectra were recorded on a Perkin Elmer FT-IR 410 spectrometer in KBr. ^{1}H and ^{13}C NMR spectra were recorded using Bruker Avance-300 (300 MHz) NMR

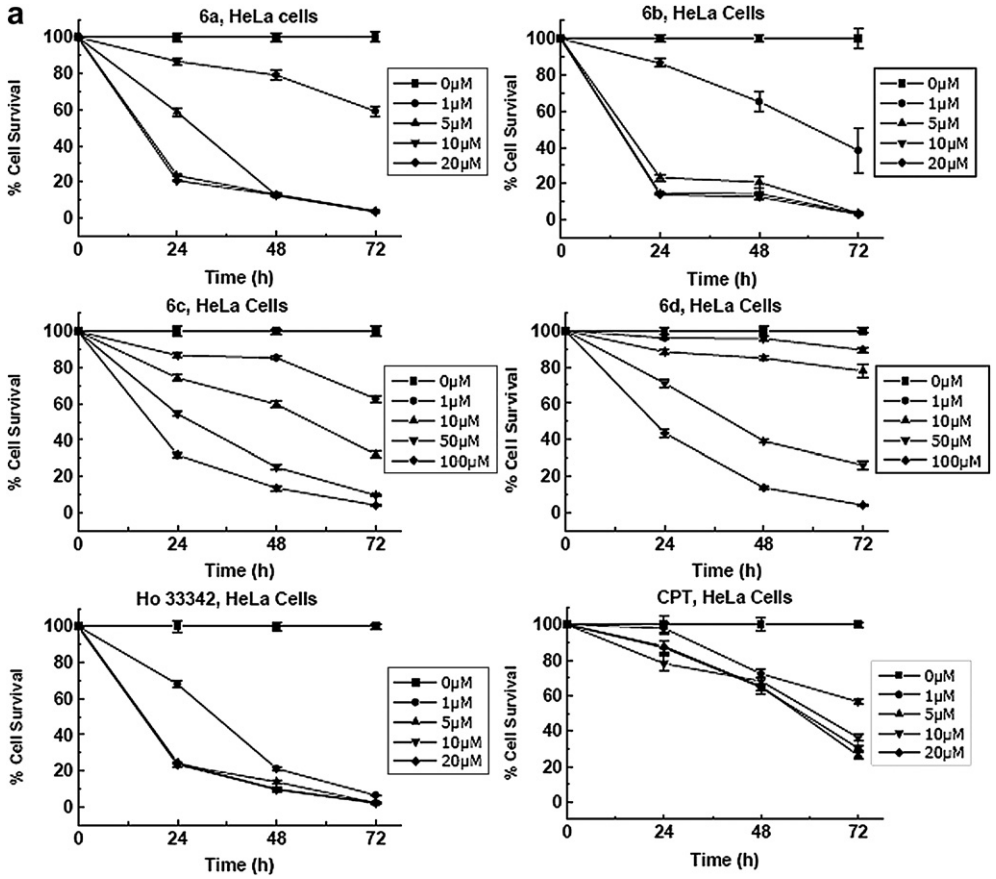


Fig. 4. a. Antiproliferative effects of the bisbenzimidazole derivatives **6a**–**d**, **Hoechst 33342** on HeLa cell line. b. Antiproliferative effects of the bisbenzimidazole derivatives **6a**–**d**, **Hoechst 33342** on MCF7 cell line. c. Antiproliferative effects of the bisbenzimidazole derivatives **6a**–**d**, **Hoechst 33342** on U87 cell line.

spectrometer. Mass spectra were recorded on ESI-TOF-MS-MS mass spectrometer (Applied Biosystems). Elemental analysis was done on GmbH VarioeEL elemental analyser.

*5.1.1. 4-Cyano-1,2-phenylenendiamine (**1**)*

A solution of 4-amino-3-nitrobenzonitrile (0.52 g, 3.22 mmol) in ethanol was treated with 10% Pd/C (140 mg), and mixture was hydrogenated at rt at 42 psi pressure of hydrogen using Parr hydrogenator (Parr Instrument Company, Illinois, USA). The reaction was followed by TLC. After 30 min, the expected volume of hydrogen had been taken up (22 psi) and reaction mixture was colorless. The reaction mixture was filtered through Celite bed and filtrate was used for next step without further purification; $R_f = 0.52$ in 100% EtOAc.

5.1.2. General procedure for preparation of 2-aryl-5-cyanobenzimidazole

*5.1.2.1. 5-Cyano-2-(2,4-difluorophenyl)benzimidazole (**2a**).* To a methanolic solution of the hydrogenated product **1** (~0.40 g, ~3.06 mmol), equivalent amount of 2,4-difluorobenzaldehyde (0.44 mL, 3.06 mmol) and half equivalent of aqueous solution of $Na_2S_2O_5$ (0.29 g, 1.53 mmol in 3 mL H_2O) were added. The resulting solution was stirred at reflux for 5 h, and filtered through a bed of Celite. The filtrate was concentrated under reduced pressure and residue was purified by column chromatography (eluent pet. ether/EtOAc, 7:3). The pure product was isolated as a solid (0.46 g, 1.83 mmol 65% yield); $R_f = 0.73$ in 100% EtOAc; mp = 256.5–258.3 °C; FTIR (KBr, cm^{-1}): 3328.68, 2229.86, 1623.70, 1480.75, 808.92, 664.58; ^{1}H NMR (300 MHz, DMSO) δ ppm = 7.32 (m, 1H, Ar–H), 7.54 (m, 1H, Ar–H), 7.61 (d, 1H, Ar–H, $J = 8.4$), 7.77 (d, 1H, Ar–H, $J = 7.8$), 8.154 (br s, 1H, Ar–H), 8.28 (m, 1H); ESI-MS: *m*/*z* found 254.0976 (M − H); exact mass 255.06. Anal. Calcd. for $C_{14}H_7F_2N_3$: C, 65.88; H, 2.76; N, 16.46. Found: C, 65.82; H, 2.78; N, 16.52.

*5.1.2.2. 5-Cyano-2-(2,4-dichlorophenyl)benzimidazole (**2b**).* To a ethanolic solution of the hydrogenated product **1** (~0.40 g, ~3.06 mmol), equivalent amount of 2,4 dichlorobenzaldehyde (0.53 g, 3.06 mol) and half equivalent of aqueous solution of $Na_2S_2O_5$ (0.29 g, 1.53 mmol in 3 mL H_2O) was treated as described for **2a** and the residue obtained was purified by column chromatography (eluent pet. ether/EtOAc = 5:5). The pure product was isolated as solid (0.65 g, 2.29 mmol 75% yield); $R_f = 0.65$ in 100% EtOAc; mp = 233.6–234.8 °C; FTIR (KBr, cm^{-1}): 3294.28, 2230.73, 1590.20, 1413.56, 808.45, 625.50; ^{1}H NMR (300 MHz, DMSO) δ ppm = 7.62 (s, 1H, Ar–H), 7.65 (s, 1H, Ar–H), 7.79 (d, 1H, Ar–H, $J = 8.4$), 7.87 (s, 1H, Ar–H), 7.94 (d, 1H, Ar–H, $J = 8.4$), 8.20 (s, 1H, Ar–H), 13.07 (br s, exch, 1H, NH); ESI-MS: *m*/*z* found 285.7627 (M − H); exact mass 287.00. Anal. Calcd. for $C_{14}H_7Cl_2N_3$: C, 58.36; H, 2.45; N, 14.58. Found: C, 58.40; H, 2.38; N, 14.64.

*5.1.2.3. 5-Cyano-2-(3,4-dimethoxyphenyl)benzimidazole (**2c**).* To a ethanolic solution of the hydrogenated product **1** (~0.40 g, ~3.06 mmol), equivalent amount of 3,4 dimethoxybenzaldehyde (0.50 g, 3.06 mmol) and half equivalent of solution of $Na_2S_2O_5$ (0.29 g, 1.53 mmol in 3 mL H_2O) were added. The resulting solution was treated as described for **2a** and the obtained residue was purified by column chromatography (fluent pet ether/EtOAc = 2:8). The pure product was isolated as light solid (0.68 g, 2.44 mmol 80%

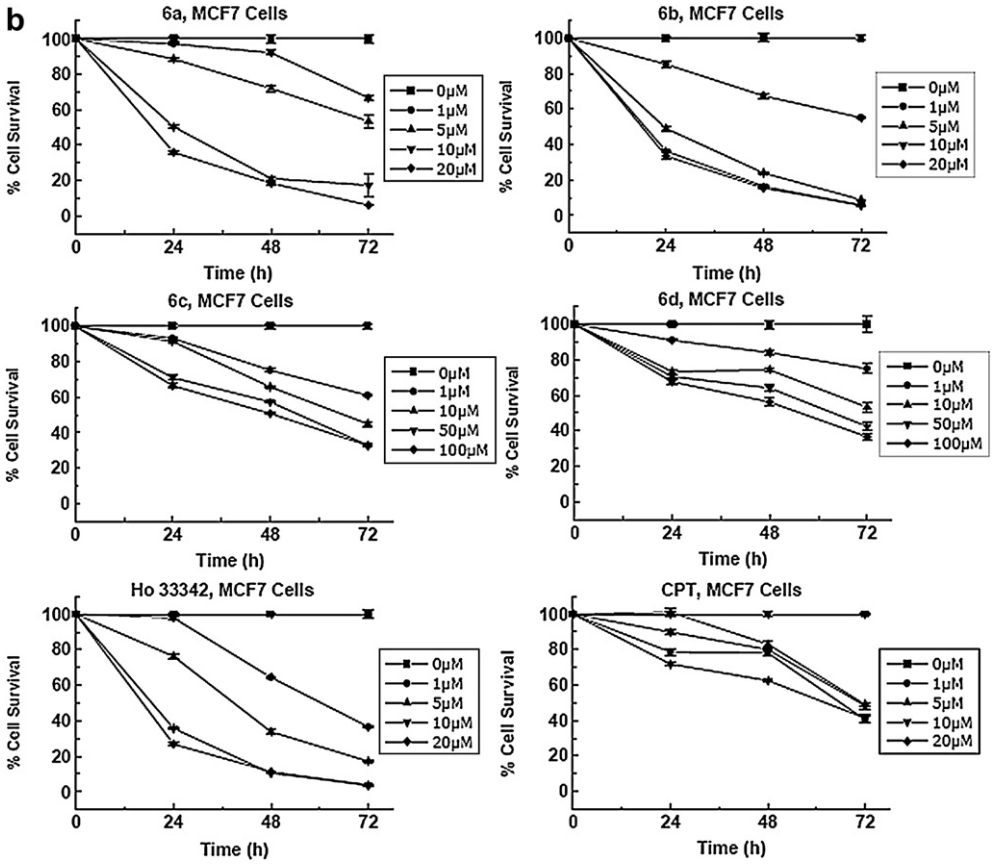


Fig. 4. (*continued*).

yield); $R_f = 0.48$ in 100% EtOAc; mp = 223.1–224.8 °C; FTIR (KBr, cm^{-1}): 3437.66, 2220.87, 1502.97, 1267.03, 1022.70, 813.60; ^1H NMR (300 MHz, DMSO) δ ppm = 3.83 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 7.13 (d, 1H, Ar–H, $J = 8.4$), 7.54 (d, 1H, Ar–H, $J = 8.4$), 7.69 (d, 1H, Ar–H, $J = 7.8$), 7.77 (s, 2H, Ar–H), 8.07 (s, 1H, Ar–H), 13.29 (br s, exch, 1H, NH); ESI-MS: *m*/*z* found 279.882 (M + H); exact mass 279. Anal. Calcd. for $C_{16}H_{13}N_3O_2$: C, 68.81; H, 4.69; N, 15.05. Found: C, 68.78; H, 4.65; N, 15.14.

*5.1.2.4. 5-Cyano-2-(3,4,5-trimethoxyphenyl)benzimidazole (**2d**).* To a ethanolic solution of the hydrogenated product **1** (~0.40 g, ~3.06 mmol), equivalent amount of 3,4,5 trimethoxybenzaldehyde (0.60 g, 3.06 mmol) and half equivalent of aqueous solution of $Na_2S_2O_5$ (0.29 g, 1.53 mmol in 3 mL H_2O) were added. The resulting solution was treated as described for **2a** and the obtained residue was purified by column chromatography (eluent pet ether/EtOAc = 3:7). The pure product was isolated as solid (0.80 g, 2.60 mmol 85% yield); $R_f = 0.52$ in 100% EtOAc; mp = 229.3–230.9 °C; FTIR (KBr, cm^{-1}): 3435.99, 2222.98, 1590, 1465.25, 1239.69, 1127.10; ^1H NMR (300 MHz, DMSO) δ ppm = 3.73 (s, 3H, OCH_3), 3.89 (s, 6H, OCH_3), 7.56 (m, 3H, Ar–H), 7.74 (br s, Ar–H), 8.14 (br s, 1H, Ar–H), 13.39 (br s, exch, 1H, NH); ESI-MS: *m*/*z* found 310.0189 (M + H); exact mass 309.11. Anal. Calcd. for $C_{17}H_{15}N_3O_3$: C, 66.01; H, 4.89; N, 13.58. Found: C, 66.05; H, 4.92; N, 13.54.

5.1.3. General method of the conversion of 5-cyanobenzimidazole to 5-formylbenzimidazoles

*5.1.3.1. 5-Formyl-2-(2,4-difluorophenyl)benzimidazole (**3a**).* Using the similar procedure to that described previously [38] Ni–Al alloy (1.25 g) was added to a solution of **2a** (0.30 g, 1.17 mmol) in formic acid (18 mL) and H_2O (6.5 mL). The reaction mixture was heated at 95 °C for 5 h under N_2. The hot mixture was filtered through a bed of Celite and the reaction flask and the Celite bed were rinsed with water. The aqueous solution was concentrated to dryness. After addition of H_2O to this residue, a white precipitate formed. The pH of this suspension was adjusted to 9 by the dropwise addition of 2 N NaOH and the product was then extracted through EtOAc. The organic layer was dried over anhydrous Na_2SO_4, then the solvent was removed and column chromatography using silica gel was done. The pure product was eluted out in 30–40% EtOAc as solid (0.166 g, 0.64 mmol, 55% yield); $R_f = 0.60$ in 100% EtOAc; mp = 214.5–216 °C; FTIR (KBr, cm^{-1}): 3223.86, 1682.48, 1626.35, 1482.26, 1289.41, 1148.91, 806.85, 639.16; ^1H NMR (300 MHz, DMSO) δ ppm = 7.32 (m, 1H, Ar–H), 7.56 (m, 1H, Ar–H), 7.78 (m, 2H, Ar–H), 8.14 (s, 1H, Ar–H), 8.28 (m, 1H, Ar–H), 10.07 (s, 1H, CHO), 13.07 (s, 1H, NH); ESI-MS: *m*/*z* found 259.3453 (M + H); exact mass 258.06. Anal. Calcd. for $C_{14}H_8F_2N_2O$: C, 65.12; H, 3.12; N, 10.85. Found: C, 65.06; H, 3.14; N, 10.89.

*5.1.3.2. 5-Formyl-2-(2,4-dichlorophenyl)benzimidazole (**3b**).* A reaction mixture of Ni–Al alloy (1.25 g) and **2b** (0.30 g, 1.04 mmol) in formic acid (18 mL) and H_2O (6.5 mL) was treated as **3a**. Purification by column chromatography using gradient of 50–60% EtOAc gave solid (0.17 g, 0.58 mmol, 56% yield); $R_f = 0.56$ in 100% EtOAc; mp = 208–210.5 °C; FTIR (KBr, cm^{-1}): 3360.34, 1685.59, 1615.03, 1410.36, 1313.21, 1152.34, 1152.34, 812.71, 788.04, 630.42; ^1H NMR (300 MHz, DMSO) δ ppm = 7.65 (d, 1H, Ar–H, $J = 8.3$), 7.82 (s, 2H, Ar–H), 7.88 (s, 1H, Ar–H), 7.97 (d, 2H, Ar–H, $J = 8.3$), 8.25 (s, 1H, Ar–H), 10.08 (s, 1H, CHO), 13.24 (br s, exch, 1H, NH); ESI-MS: *m*/*z* found 291.0148 (M + H); exact mass 290. Anal. Calcd. for $C_{14}H_8Cl_2N_2O$: C, 57.76; H, 2.77; N, 9.62. Found: C, 57.70; H, 2.82; N, 9.56.

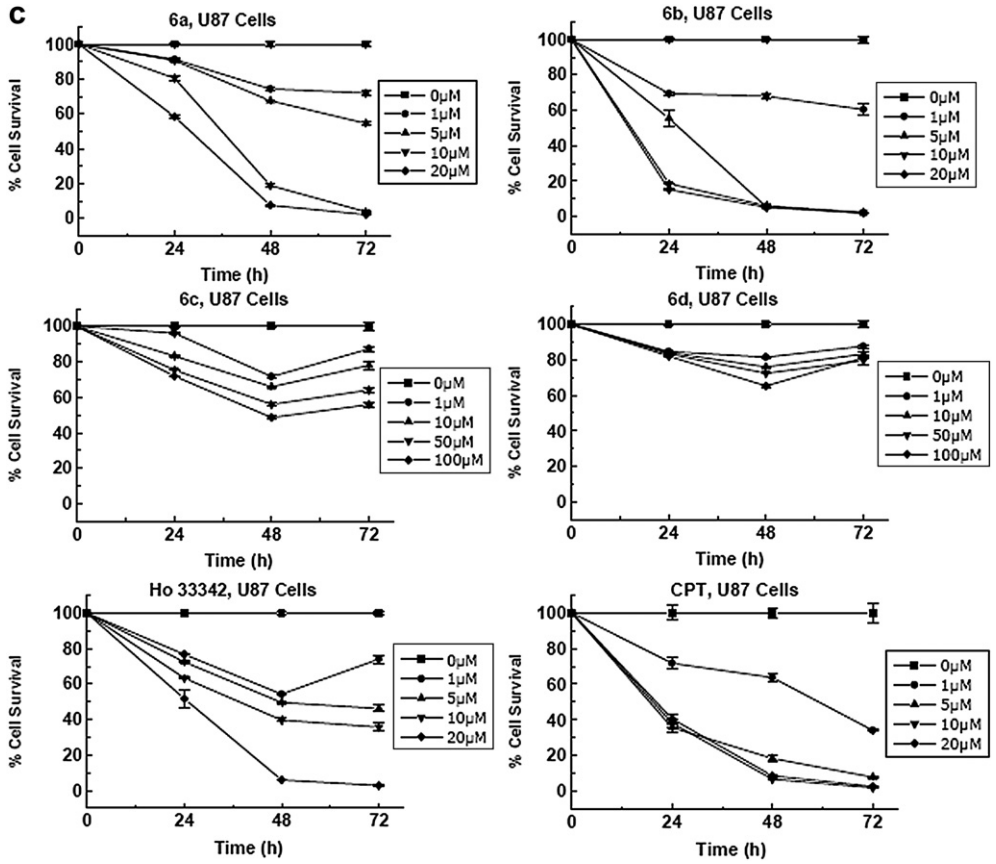


Fig. 4. (*continued*).

*5.1.3.3. 5-Formyl-2-(3,4-dimethoxyphenyl)benzimidazole (**3c**).* A reaction mixture of Ni–Al alloy (2.50 g) and **2c** (0.58 g, 2.08 mmol) in formic acid (36 mL) and H_2O (14 mL) was treated as **3a**. Purification by column chromatography using gradient of EtOAc to 4% MeOH/EtOAc gave solid (0.35 g, 1.25 mmol, 60% yield); $R_f = 0.40$ in 100% EtOAc; mp = 232.6–235.4 °C; FTIR (KBr, cm^{-1}): 3429.26, 1686.19, 1619.11, 1505.75, 1265.35 1139.52, 1020.75, 810.45; ^1H NMR (300 MHz, DMSO) δ ppm = 3.85 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 7.15 (d, 1H, Ar–H, $J = 8.3$), 7.54 (d, 1H, Ar–H, $J = 8.4$), 7.69 (d, 1H, Ar–H, $J = 7.8$), 7.77 (s, 2H, Ar–H), 8.07 (s, 1H, Ar–H), 10.05 (s, 1H, CHO), 13.29 (br s, exch, 1H, NH); ESI-MS: *m/z* found 282.9404 (M + H); exact mass 282.10. Anal. Calcd. for $C_{16}H_{14}N_2O_3$: C, 68.07; H, 5.00; N, 9.92. Found: C, 68.01; H, 4.91; N, 9.88.

*5.1.3.4. 5-Formyl-2-(3,4,5-trimethoxyphenyl)benzimidazole (**3d**).* A reaction mixture of Ni–Al alloy (2.50 g) and **2d** (0.642 g, 2.08 mmol) in formic acid (36 mL) and H_2O (14 mL) was treated as **3a**. Purification by column chromatography using gradient of EtOAc to 2% MeOH/EtOAc and the product was obtained as solid (0.389 g, 1.24 mmol, 60% yield); $R_f = 0.47$ in 100% EtOAc; mp = 214.2–215.4 °C; FTIR (KBr, cm^{-1}): 3410.47, 1688.95, 1589.74, 1463.65, 1426.56, 1127.85, 1005.82, 787.58; ^1H NMR (300 MHz, DMSO) δ ppm = 3.75 (s, 3H, OCH_3), 3.92 (s, 6H, OCH_3), 7.56 (s, 2H, Ar–H), 7.77 (s, 2H, Ar–H), 8.17 (br s, 1H, Ar–H), 10.06 (s, 1H, CHO), 13.33 (br s, exch, 1H, NH); ESI-MS: *m/z* found 313.1179 (M + H); exact mass 312.12. Anal. Calcd. for $C_{17}H_{16}N_2O_4$: C, 65.38; H, 5.16; N, 8.97. Found: C, 65.32; H, 5.20; N, 8.91.

Table 2
IC_{50} of the synthesized compounds against U87, MCF7 and HeLa cells in vitro after 72 h of continuous exposure of compound (IC_{50} is given in μM).

Compound	U87	MCF7	HeLa
Hoechst 33342	4.2	0.5	0.3
6a	5.5	5.5	1.5
6b	1.5	1.3	0.6
6c	>100	5.3	3.4
6d	>100	16.8	29.3
Camptothecin	0.5	0.65	1.5

All values are in μM.
Values are mean of triplicates of three independent experiments.

*5.1.3.5. 5-(4-Methyl-1-piperazinyl)-2-nitroaniline (**4**).* 5-Chloro-2-nitroaniline (2 g, 11.6 mmol), 4-methylpiperazine (1.53 mL, 13.8 mmol) and K_2CO_3 (2.5 g, 18 mmol) in 5 mL DMF was heated at 110 °C for 26–30 h till TLC showed the disappearance of 5-chloro-2-nitroaniline. The crude compound was suspended in water and the product was extracted with ethyl acetate. The organic layer was washed twice with water, dried over anhydrous Na_2SO_4 and concentrated. The product was purified by column chromatography using a gradient of 20% MeOH/EtOAc. The pure product was isolated as a bright yellow solid (2.32 g, 9.86 mmol 85% yield); $R_f = 0.27$ in methanol/EtOAc, 4:6; mp = 155.8–157.8 °C; FTIR (KBr, cm^{-1}): 3442.03, 3283.01, 1619.30, 1619.30, 1247.39, 1000.30, 809.79, 670.72; ^1H NMR (300 MHz, DMSO) δ ppm = 2.18 (s, 3H, N-CH_3), 2.38 (s, 4H, CH_2), 3.29 (s, 4H, CH_2), 6.24 (s, 1H, Ar–H), 6.32 (d, 1H, Ar–H, $J = 9.4$), 6.97 (br s, exch, 2H, NH_2), 7.79 (d, 1H, Ar–H, $J = 9.0$); ^{13}C NMR (75 MHz, DMSO) δ ppm: 45.50, 46.11, 54.08, 97.65, 105.34, 123.04,127.18,148.37, 154.97; ESI-MS: *m/z* found 237.1202 (M + H); exact mass 236.13. Anal. Calcd. for $C_{11}H_{16}N_4O_2$: C, 55.92; H, 6.83; N, 23.71. Found: C, 55.82; H, 6.87; N, 23.7.

*5.1.3.6. 5-(4-Methyl-1-piperazinyl)-1,2-phenylenediamine (**5**).* A solution of 5-(4-methyl-1-piperazinyl)-2-nitroaniline (0.2 g, 0.84 mmol)

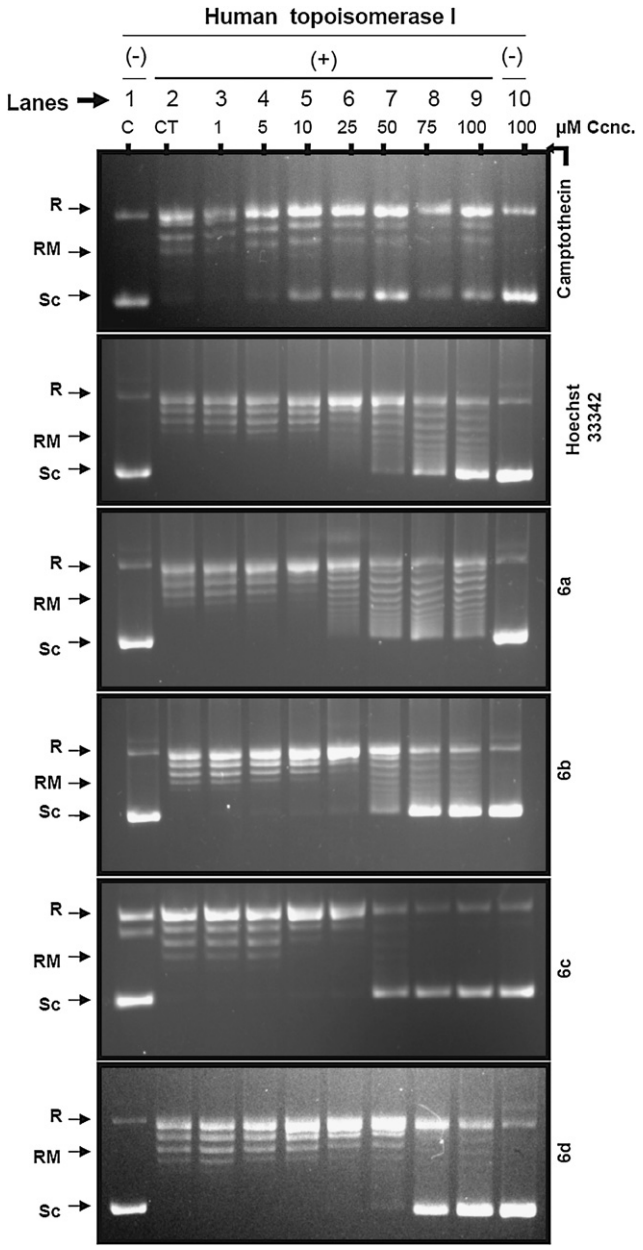


Fig. 5. Effects of DNA binding compounds on human topoisomerase I. Purified human topoisomerase I was incubated with pBS (SK+) plasmid 500 ng in the presence and absence of compounds **Hoechst 33342**, **6a**, **6b**, **6c**, **6d** and **Camptothecin** at 37 °C. Lane 1, pBS (SK+) plasmid; lane 2, plasmid + human topoisomerase I, two units; lanes 3–9, incubation of plasmid with 1, 5, 10, 25, 50, 75 and 100 μM of compound (**Camptothecin** or **Hoechst 33342** or **6a**–**d**) for 30 min at 37 °C with simultaneous addition of two units of human topoisomerase I; lane 10, incubation of plasmid with 100 μM of compound without addition of topoisomerase I.

in ethanol was treated with 10% Pd/C (130 mg) and mixture was hydrogenated at rt under 42 psi H_2 pressure. The reaction was followed by TLC. After 2 h, the expected volume of hydrogen had been taken up (14 psi) and reaction mixture was colorless. Reaction mixture was filtered through Celite and filtrate was used for next step without further purification; R_f = 0.14 in 40% methanol in EtOAc.

5.1.4. General synthesis of 2-aryl-5-[5-(4-methylpiperazin-1-yl)-1H-benzimidazol-2-yl]-1H-benzimidazole

*5.1.4.1. 2-(2,4-Difluorophenyl)-5-[5-(4-methylpiperazin-1-yl)-1H benzimidazol-2-yl]-1H-benzimidazole (**6a**).* To a ethanolic solution of the hydrogenated product **5** (~0.15 g, ~0.77 mmol), equivalent amount of 2-(2,4-difluorophenyl)-5-formylbenzimidazole (0.20 g, 0.77 mmol) and half equivalent of solution of $Na_2S_2O_5$ (0.072 g, 0.38 mmol in 1 ml H_2O) were added. The resulting solution was stirred at reflux for 6 h, then cooled to room temperature and filtered through a bed of Celite. The filtrate was concentrated under reduced pressure and the obtained residue was purified by column chromatography using a gradient of 60–80% MeOH/EtOAc. The pure product was isolated as an orange solid (0.215 g, 0.48 mmol 63% yield); R_f = 0.29 in 100% MeOH; mp = 234.1–235.8 °C; FTIR (KBr, cm^{-1}): 3421.67, 1628,57, 1447.68, 1267.23, 1142.34, 1010.00, 973.64, 817.86; ^{1}H NMR (300 MHz, DMSO) δ ppm = 2.43 (s, 3H, CH_3), 2.77 (s, 4H, CH_2), 3.21 (s, 4H, CH_2), 6.95 (d, 1H, Ar–H, J = 8.2), 7.05 (s, 1H, Ar–H), 7.33 (m, 1H, Ar–H), 7.47 (d, 1H, Ar–H, J = 8.2), 7.53 (m, 1H, Ar–H), 7.75 (br s, 1H, Ar–H), 8.07 (br s, 1H, Ar–H), 8.31 (d, 1H, Ar–H, J = 6.9), 8.36 (d, 1H, Ar–H, J = 6.0), 12.88 (br s, exch, 1H, NH); ^{13}C NMR (75 MHz, DMSO) δ ppm = 44.59, 49.13, 54.13, 105.06, 109.74, 112.6, 113.79, 114.76,116.43, 119.06, 120.8, 124.9, 131.8, 147.1, 151.4, 158.0, 161.4, 164.8; ESI-MS: m/z found 445.1476 (M + H); exact mass 444.19. Anal. Calcd. for $C_{25}H_{22}F_2N_6$: C, 67.55; H, 4.99; N, 18.91. Found: C, 67.61; H, 4.95; N, 18.87.

*5.1.4.2. 2-(2,4-Dichlorophenyl)-5-[5-(4-methylpiperazin-1-yl)-1H-benzimidazol-2-yl]-1H-benzimidazole (**6b**).* The ethanolic solution of the hydrogenated product **5** (~0.21 g, ~1.03 mmol), equivalent amount of 2-(2,4-dichlorophenyl)-5-formylbenzimidazole (0.30 g, 1.03 mmol) and half equivalent of solution of $Na_2S_2O_5$ (0.097 g, 0.515 mmol in 1.5 mL H_2O) were treated as described for **6a** and the obtained residue was purified by column chromatography using a gradient of 70–90% MeOH/EtOAc. The pure product was isolated as a yellow solid (0.314 g, 0.66 mmol 65% yield); R_f = 0.24 in 100% MeOH; mp = 189.8–194.2 °C; FTIR (KBr, cm^{-1}): 3434.62, 1629.81, 1587.09, 1422.04, 1286.88, 1143.47, 1102.72, 1047.72, 820.09; ^{1}H NMR (300 MHz, DMSO) δ ppm = 2.24 (s, 3H, CH_3), 2.50 (s, 4H, CH_2), 3.12 (s, 4H, CH_2), 6.94 (d, 1H, Ar–H, J = 8.2), 7.02 (s, 1H, Ar–H), 7.44 (s, 1H, Ar–H), 7.64 (d, 1H, Ar–H, J = 7.8), 7.78 (s, 1H, Ar–H), 7.87 (s, 1H, Ar–H), 7.98 (d, 1H, Ar–H, J = 8.16), 8.09 (d, 1H, Ar–H, J = 6.6), 8.40 (s, 1H, Ar–H), 12.65 (br s, exch, 1H, NH); ^{13}C NMR (75 MHz, DMSO) δ ppm = 45.7, 49.9, 54.8, 113.7, 116.3, 118.6, 121.08, 127.7, 128.6, 129.9, 132.7, 133.3, 135.2, 147.7, 149.5, 151.2; ESI-MS: m/z found 476.9579 (M + H); exact mass 476.13. Anal. Calcd. for $C_{25}H_{22}Cl_2N_6$: C, 62.90; H, 4.64; N, 17.60. Found: C, 62.94; H, 4.60; N, 17.56.

*5.1.4.3. 2-(3,4-Dimethoxyphenyl)-5-[5-(4-methylpiperazin-1-yl)-1H-benzimidazol-2-yl]-1H-benzimidazole (**6c**).* The ethanolic solution of the hydrogenated product **5**(~0.36 g, ~1.77 mmol), equivalent amount of 2-(3,4-dimethoxyphenyl)-5-formylbenzimidazole (0.50 g, 1.77 mmol) and half equivalent of solution of $Na_2S_2O_5$ (0.168 g, 0.885 mmol in 3 mL H_2O) were treated as described for **6a** and the obtained residue was purified by column chromatography using a gradient of 100% MeOH. The pure product was isolated as a brown orange solid (0.51 g, 1.09 mmol 62% yield); R_f = 0.15 in 100% MeOH; mp = 253.7–254.8 °C; FTIR (KBr, cm^{-1}): 3390.72, 1635.70, 1500.18, 1437.35, 163.87, 1226.25, 1146.13, 1021.17, 981.73, 811.22, 627.63; ^{1}H NMR (300 MHz, DMSO) δ ppm = 1.77 (s, 3H, CH_3), 2.21 (s, 4H, CH_2), 2.49 (s, 4H, CH_2), 3.83 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 6.91 (d, 1H, Ar–H, J = 8.7), 6.92 (s, 1H, Ar–H), 7.12 (d, 1H, Ar–H, J = 8.1), 7.43 (d, 1H, Ar–H, J = 8.7), 7.67 (d, 1H, Ar–H, J = 8.4), 7.82 (m, 2H, Ar–H), 8.00 (d, 1H, Ar–H, J = 8.4), 8.31 (s, 1H, Ar–H), 12.91 (br s, exch, 1H, NH); ^{13}C NMR (75 MHz, DMSO) δ ppm = 45.8, 49.9, 54.9, 55.6, 109.9, 111.7, 119.6, 120.6, 122.61, 124.4, 147.6, 148.9, 150.4, 151.6, 153.06; ESI-MS: m/z found 469.25 (M + H); exact mass 468.23. Anal. Calcd. for $C_{27}H_{28}N_6O_2$: C, 69.21; H, 6.02; N, 17.94. Found: C, 69.25; H, 6.06; N, 17.89.

*5.1.4.4. 2-(3,4,5-Trimethoxyphenyl)-5-[5-(4-methylpiperazin-1-yl)-1H-benzimidazol-2-yl]-1H-benzimidazole (**6d**).* The ethanolic solution of the hydrogenated product **5** (~0.26 g, ~1.28 mmol), equivalent amount of 2-(3,4,5-trimethoxyphenyl)-5-formylbenzimidazole (0.40 g, 1.28 mmol) and half equivalent of solution of $Na_2S_2O_5$ (0.121 g, 0.64 mmol in 2 ml H_2O) were treated as described for **6a** and the obtained residue was purified by column chromatography using a gradient of 80–95% MeOH/EtOAc. The pure product was isolated as solid (0.433 g, 0.87 mmol 68% yield); $R_f = 0.21$ in 100% MeOH, mp = 260.2–262.5 °C; FTIR (KBr, cm^{-1}): 3400.50, 1591.16, 1464.32, 1426.86, 1238.37, 1127.55, 997.76, 727.09; ^{1}H NMR (300 MHz, DMSO) δ ppm = 1.18 (s, 3H, CH_3), 2.26 (s, 4H, CH_2), 2.53 (s, 4H, CH_2), 3.75 (s, 3H, OCH_3), 3.93 (s, 6H, OCH_3), 6.95 (d, 1H, Ar–H, $J = 6$), 7.03 (s, 1H, Ar–H), 7.46 (d, 1H, Ar–H, $J = 8.2$), 7.60 (s, 1H, Ar–H), 7.72 (d, 1H, Ar–H, $J = 4.5$), 8.04 (d, 1H, Ar–H, $J = 5.4$), 8.34 (s, 2H, Ar–H); ^{13}C NMR (75 MHz, DMSO) δ ppm = 45.5, 49.8, 54.7, 56.1, 60.1, 100.3, 104.1, 112.4, 113.6, 115.4, 120.9, 124.6, 125.2, 139.1, 147.6, 151.4, 152.7, 153.2, 164.5; ESI-MS: *m/z* found 499.1372 (M + H); exact mass 498.24. Anal. Calcd. for $C_{28}H_{30}N_6O_3$: C, 67.45; H, 6.06; N, 16.86. Found: C, 67.49; H, 5.98; N, 16.91.

5.2. Biological investigation

5.2.1. Materials

All cell culture reagents were obtained from HIMEDIA (India). DMEM, **Hoechst 33342**, Camptothecin (CPT) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemicals (St Louis, MO). The human cancerous cell lines U87, HeLa and MCF7 were procured from National Center for Cell Science, Pune, India. An extinction coefficient of 6420 $M^{-1}\,cm^{-1}$ was used to measure the nucleotide concentration of CT-DNA (Calf thymus DNA, Pharmacia) solution. The stock solution of bisbenzimidazoles was made at 10 mM concentration in methanol. The molar extinction coefficients were found to be $\varepsilon_{334} = 22{,}600$ for **6a**, $\varepsilon_{334} = 23{,}800$ for **6b**, $\varepsilon_{340} = 26{,}200$ for **6c**, $\varepsilon_{340} = 24{,}400$ for **6d** and $\varepsilon_{340} = 42{,}000$ $M^{-1}\,cm^{-1}$ for **Hoechst** in methanol at 25 °C. Working stocks were prepared fresh before each experiment from these by dilution with buffer (20 mM sodium cacodylate buffer, 100 mM NaCl, pH 7.2).

5.2.2. Absorption titration

Drug–DNA interaction studies were done using spectroscopic technique and their binding pattern was determined by the changes in their absorption spectra. Increasing concentration of CT-DNA (5, 10, 15, 20 μM) was titrated into a fixed concentration of compounds (**6a**–**d** and **Hoechst**) i.e., 5 μM in 20 mM sodium cacodylate buffer, 100 mM NaCl, pH 7.2. These measurements were performed at DNA/drug ratio, $r = 1$–4. Absorption spectra were recorded by UV–vis Spectrophotometer (Cary Varian 300). Drug–DNA solution was equilibrated for 15 min and spectral measurements were recorded using 1 cm path length quartz cell at 25 °C.

5.2.3. Fluorescence measurement

Equilibrium binding experiments were conducted using a on a Fluoromax-4 (JobinYvon) instrument at temperature 25 °C. A solution of ligand **6a**–**d** and **Hoechst** (100 nm concentrations) was excited at its respective λ_{max} (slits of 5 nm), and resulting emission curves (from 350 to 650 nm) were recorded after serial additions of a concentrated CT-DNA solution [0.1–40 μM]. After each addition, the solution was mixed by pipetting up and down. Sample equilibrium was monitored by continually exciting and scanning the sample at different times and was usually reached within 5 min.

5.2.4. Cell culture

Cells were routinely maintained in DMEM (D-7777) medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 5 U mL^{-1} insulin, 100 U mL^{-1} streptomycin, 100 μg mL^{-1} penicillin and 45 μg mL^{-1} gentamicin. The cells were grown at 37 °C, 5% CO_2, 95% air and 84% relative humidity for 24 h prior to addition of experimental compounds. The final concentration of methanol was 1% in the culture medium (v/v) (Methanol at this concentration did not affect the viability of the cells).

5.2.5. MTT assay for cell viability

Various human tumor cells (U87, HeLa and MCF7) were maintained as monolayer at 37 °C in 5% CO_2 using DMEM medium. Approximately 3000–8000 cells/well were seeded in 96-well plates containing 200 μL of medium and incubated for 24 h. The culture medium was replaced by fresh medium containing 1, 5, 10 and 20 μM concentration of **6a**, **6b**, **Hoechst**, **Camptothecin** and 1, 10, 50, 100 μM of **6c** and **6d** and incubated for 24, 48 and 72 h. The cell viability was determined by the MTT assay following the procedure described by Price and McMillan [39]. The light absorbance was measured at 570 nm wavelength using a microplate reader (Infinite M200; Tecan Group Ltd., Männedorf, Switzerland).

5.2.6. Plasmid relaxation assay

The type IB DNA topoisomerase was assayed by decreasing mobility of the relaxed isomers of supercoiled pBS(SK +) DNA in an agarose gel. The reaction mixture (25 μL) contained 25 mM Tris HCl, pH 7.5, 5% glycerol, 50 mM KCl, 0.5 mM DTT, 10 mM $MgCl_2$, 30 μg mL^{-1} BSA, 0.5 μg {pBSK II(+)} two units of enzyme (one unit is defined as the amount of enzyme required to convert 50% of 0.5 μg supercoiled DNA substrate to the relaxed form under standard assay conditions). Reactions were carried out at 37 °C for 30 min with an increasing concentration of **6a**–**d** and **Hoechst** (0, 1, 5, 10, 25, 50, 75, 100 μM) and then terminated by adding 10 mM EDTA, 0.5% SDS, 0.25 μg mL^{-1} bromophenol blue and 15% glycerol. The samples were electrophoresed in a horizontal 1% agarose gel in trisborate/EDTA buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8) at 1.5 V/cm for 14–16 h at rt. DMSO or Methanol concentrations in each reaction were maintained at 1% by the addition of serially diluted drug stocks so as not to produce solvent mediated inhibition of topoisomerase I. The gels were stained with ethidium bromide (5 μg mL^{-1}), destained in water and photographed under UV illumination at alpha imager 2200.

Acknowledgements

Funding: This work was funded by the Swedish International Development Agency (Sweden) and Department of Biotechnology (India). MS received a Junior Research Fellowship from the Department of Science and Technology and Senior Research Fellowship from Council of Scientific and Industrial Research (New Delhi, India).

References

[1] M. Lee, V.L. Rhodens, M.D. Wyatt, S. Forrow, J.A. Hartley, Anti-Cancer Drug Des. 8 (1993) 173–193.
[2] M. D'Incalci, Ann. Oncol. 5 (1994) 877–878.
[3] L.H. Hurley, V.L. Reynolds, D.H. Swenson, G.L. Petzold, T.A. Scahill, Science 226 (1984) 843–844.
[4] V.L. Reynolds, I.J. Molineux, D.J. Kaplan, D.H. Swenson, L.H. Hurley, Biochemistry 24 (1985) 6228–6237.
[5] M. Broggini, H.M. Coley, N. Mongelli, E. Pesenti, M.D. Wyatt, J.A. Hartley, M. D'Incalci, Nucleic Acids Res. 23 (1995) 81–87.
[6] D. Sun, L.H. Hurley, Biochemistry 31 (1992) 2822–2829.
[7] S. Marchini, P. Cozzi, I. Beria, C. Geroni, L. Capolongo, M. D'Incalci, M. Broggini, Anti-Cancer Drug Des. 13 (1998) 193–205.

[8] A.Y. Chen, Y. Chiang, B. Gatto, L.F. Liu, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 8131–8135.
[9] K.J. Soderlind, B. Gorodetsky, A.K. Singh, N.R. Bachur, G.G. Miller, J.W. Lown, Anti-Cancer Drug Des. 14 (1999) 19–36.
[10] J.J. Champoux, In: N.R. Cozzarelli, J.C. Wang (Eds.), DNA Topology and its Biological Effects, Cold Spring Harbor Laboratory Press, Plainview, NY, 1990, pp. 217–242.
[11] J.C. Wang, Annu. Rev. Biochem. 65 (1996) 635–692.
[12] J.J. Champoux, Annu. Rev. Biochem. 70 (2001) 369–413.
[13] J.C. Wang, Nat. Rev. Mol. Cell Biol. 3 (2002) 430–440.
[14] C. Jaxel, M. Nadal, G. Mirambeau, P. Forterre, M. Takahashi, M. Duguet, EMBO J. 8 (1989) 3135–3139.
[15] S. Brahms, S. Nakasu, A. Kikuchi, J.G. Brahms, Eur. J. Biochem. 184 (1989) 297–303.
[16] C. Cheng, S. Shuman, J. Biol. Chem. 273 (1998) 11589–11595.
[17] L.F. Liu, K.G. Miller, Proc. Natl. Acad. Sci. U.S.A. 78 (1981) 3487–3491.
[18] T. Goto, P. Laipis, J.C. Wang, J. Biol. Chem. 259 (1984) 10422–10429.
[19] B.S.P. Reddy, S.M. Sondhi, J.W. Lown, Pharmacol. Ther. 84 (1999) 1–111.
[20] G.L. Grravatt, B.C. Buguley, R.W. Wilson, W.A. Denny, J. Med. Chem. 37 (1994) 4338–4345.
[21] E.H. Kraut, L. Masspeis, S. Bakerzak, M. Grever, Proc. Am. Soc. Clin. Oncol. 7 (1988) 62.
[22] E. Kraut, T. Fleming, M. Segal, J. Neidhart, B.C. Behrens, J. MacDonald, Invest. New Drugs 9 (1999) 95–96.
[23] G.J. Finlay, B.C. Baguley, Eur. J. Cancer 26 (1990) 586–589.
[24] P.E. Pjura, K. Grzeskoviak, R.E. Dickerson, J. Mol. Biol. 197 (1987) 257–271.
[25] M. Teng, N. Usman, C.A. Frederick, A. Wang, Nucleic Acids Res. 16 (1988) 2671–2690.
[26] S. Kumar, B. Yadagiri, J. Zimmermann, R.T. Pon, J.W. Lown, J. Biomol. Struct. Dyn. 8 (1990) 331–357.
[27] Q. Sun, B. Gatto, C. Yu, A. Liu, L.F. Liu, E.J. LaVoie, Bioorg. Med. Chem. Lett. 4 (1994) 2871–2876.
[28] S.Y. Breusegem, S.E. Sadat-Ebrahimi, K.T. Douglas, R.M. Clegg, F.G. Loontiens, J. Mol. Biol. 308 (2001) 649–663.
[29] X. Jing, Q. Zhu, F. Xu, X. Ren, Dong Li, C. Yan, Synth. Commun. 36 (2006) 2597–2601.
[30] V.K. Tandon, M. Kumar, Tetrahedron Lett. 45 (2004) 4185–4187.
[31] K. Bougrin, A. Loupy, A. Petit, B. Daou, M. Soufiaoui, Tetrahedron 57 (2001) 163–168.
[32] R. Rastogi, S. Sharma, Synthesis (1983) 861–882.
[33] D.S. Van Vliet, P. Gillespie, J.J. Scicinski, Tetrahedron Lett. 46 (2005) 6741–6743.
[34] A. Hegedus, Z. Hell, A. Potor, Synth. Commun 36 (2006) 3625–3630.
[35] N.M. Starshikov, A.F. Khim, Chem. Heterocycl. Compd. (1980) 81–85.
[36] A. Maquestiau, L. Berte, A. Mayence, J. Vanden Eynde, J. Synth. Commun. 21 (1991) 2171–2180.
[37] U. Tawar, A.K. Jain, B.S. Dwarakanath, R. Chandra, Y. Singh, N.K. Chaudhury, D. Khaitan, V. Tandon, J. Med. Chem. 46 (2003) 3785–3792.
[38] T. van Es, B. Staskun, J. Chem. Soc. (1965) 5775–5777.
[39] Price, T.J. McMillan, Cancer Res. 50 (1990) 1392–1396.