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Synthesis and Characterization of 2-substituted benzimidazoles and their evaluation as anticancer agent

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Abstract:

In this work, we report a series of benzimidazole derivatives synthesized from benzene-1,2-diamine and aryl-aldehydes at room temperature. The synthesized compounds have been characterized on the basis of elemental analysis and various spectroscopic studies viz., IR, ^{1}H- and ^{13}C- NMR, ESI-MS as well by X-ray single X-ray crystallographic study. Interaction of these compounds with CT-DNA has been examined with fluorescence experiments and showed significant binding ability**.** All the synthesized compounds have been screened for their antitumor activities against various human cancer cell lines viz., Human breast adenocarcinoma cell line (MCF-7), Human leukemia cell line (THP-1), Human prostate cancer cell lines (PC-3) and Adenocarcinomic human alveolar basal epithelial cell lines (A-549). Interestingly, all the compounds showed significant anticancer activity.

Keywords: Benzimidazoles; Crystal structure; DNA interactive studies; Anti-proliferative studies

Introduction:

Benzimidazole is a heterocyclic moiety possessing wide spectrum of biological and clinical applications due to their structural resemblance to the naturally occurring nucleotides found in living system [1-2]. Moreover, nucleus of benzimidazole is also found in variety of naturally occurring compounds such as vitamin B_{12}, structurally similar to purine bases. Therefore, benzimidazole and its derivatives represent one of the highly biologically active class of compounds possessing various biological activities [3-7], viz., antimicrobial [8-11], anti-inflammatory [12-15], antiviral [16], anthelmintic [17], anti-tumor [18], anticancer [19], antivirus [20], antihistamine [21], antihypertensive [22], antineoplastic [23], anti-analgesic [24], antiprotozoal [25], anti-hepatitis B virus activity [26]. Furthermore, they also exhibit a remarkable activity against several viruses such as HIV [27], herpes (HSV-1) [28], and RNA influenza [29]. In addition, benzimidazoles serve as ligands for asymmetric catalysts in various reactions [30]. Therefore, the synthesis of these benzimidazole-containing compounds has received considerable attention in diverse areas of chemistry, and a number of synthetic methods have been developed to uncover a variety of new reagents for their preparation [31-40]. The most commonly-used synthetic approach typically involve the condensation of o-arylene-diamines with carbonyl compounds such as aldehydes, carboxylic acids and their derivatives which often require strongly acidic conditions, sometimes combined with very high temperature or the use of microwave irradiation [31- 40]. In addition, there are several reports on benzimidazole synthesis via the reductive cyclization of o-nitroanilines with aldehydes [41], cyclization of o-nitroaniline derivatives with aryl isothiocyanates

[42, 43], and Baker's yeast reduction of 2, 4-dinitroacyl anilines [44]. In the last decade, lot of work has been reported on synthesis of various benzimidazole derivatives and their biological applications [45]. To add the novelty in the work, herein we successfully isolated the benzimidazole derivatives in high yield without using any catalyst and screened the synthesized compounds against number of cell lines viz., human breast adenocarcinoma cell line (MCF-7), human leukemia cell line (THP-1), human prostate cancer cell lines (PC-3) and adenocarcinomic human alveolar basal epithelial cell line (A-549) and we got significant results. In addition, we also characterized benzimidazole derivative **1 by** single crystal X-ray diffraction, which has not been reported before.

Experimental:

General remarks

Glassware was oven–dried overnight at 120 ºC before use. Reactions were performed under an inert atmosphere using an argon filled glove box. All the reactions were monitored by TLC analysis using Merck Silica Gel 60 F–254 thin layer plates. Column chromatography was performed on silica gel 100–200 mesh. Benzene-1,2-diamine and aryl-aldehydes were commercially obtained and used as purchased without further purification.

Instrumentation: NMR spectra were recorded with a Jeol spectrometer at 400 MHz (^{1}H–NMR) and 100 MHz (^{13}C–NMR). The chemical shifts (δ in ppm) were reported down field from tetramethylsilane (TMS, δ scale) with d_6-DMSO as resonance referenced as internal standard. Elemental analyses were performed on a Perkin Elmer 2400 Elemental Analyzer. IR spectra were obtained using Perkin Elmer FTIR–800 Model. Fluorescence measurements were carried out on a Shimadzu spectrofluorimeter model

RF-540. Mass spectrometric analysis was conducted by using ESI mode on AGILENT Technologies 6410–triple quad LC/MS instrument.

General Procedure (GP) for synthesis of 2-phenyl-1H-benzo[d]imidazole derivatives:

To a dried 100 mL round bottom flask were added benzene-1,2-diamine (540 mg, 5 mmol) and aryl-aldehyde (5.0 mmol,) in methanol (15 ml) under argon atmosphere (Scheme 1S). Then solution was stirred for 24 h. Progress of the reaction was monitored by TLC and the solvent was removed under reduced vacuum and the residue was chromatographed on silica gel column with ethyl acetate/*n*-hexane (1:1, v/v) to afford the pure product **1-3.**

2-(2-chlorophenyl)-1H-benzo[d]imidazole, 1

Benzene-1,2-diamine (540 mg, 5.0 mmol) and 2-chloroaldehyde (700 mg, 5.0 mmol) in methanol (15 mL) were reacted according to GP. The product was purified by column chromatography on silica gel (40% EtOAc in hexane) yielded **1** (1.1 g, 4.82 mol, 96.46%); m.p. = 232 ^{o}C; IR (KBr): 3427, 3061, 2882, 1639, 1460, 1242 cm^{-1}; ^{1}H-NMR (DMSO-d$_6$, 400 MHz) δ 7.20 (t, J = 8.4 Hz, 2H, A**rH**), 7.54 (d, J = 8.4 Hz, 1H, Ar**H**), 7.66 (d, J = 8.4 Hz, 1H, Ar**H**), 7.76 (d, J = 8.8 Hz, 2H, Ar**H**), 8.09 (d, J = 8.8 Hz, 2H, Ar**H**), 12.21 (s, 1H, N**H** of Indole); ^{13}C-NMR (DMSO-d$_6$, 100 MHz): δ 112.1, 119.5, 122.8, 123.5, 123.9, 128.9, 129.8, 132.6, 141.7, 150.8; [Anal. Calcd. for $C_{13}H_9N_2Cl$ (228): C, 68.28; H, 3.97; N, 12.25; Found: C, 68.39; H, 3.99; N, 12.27], ESI: $[M+H]^+$ m/z 229.68

2-(4-bromophenyl)-1H-benzo[d]imidazole 2

Benzene-1,2-diamine (540 mg, 5.0 mmol) and 4-bromoaldehyde (920 mg, 5.0 mmol) in methanol (15 mL) were reacted according to **GP**. The product was purified by column chromatography on silica gel (40 % EtOAc in hexane) yielded **2** (1.29 g, 4.74 mol, 94.85%); m.p. = 269 ^{o}C; IR (KBr): 3417, 1646, 1435, 742, 700, 587cm^{-1}; ^{1}H-NMR (DMSO-d$_6$, 400 MHz) δ 7.33 (d, J = 8.0 Hz, 2H, Ar**H**), 7.37 – 7.41 (m, 2H, Ar**H**), 7.45 – 7.49 (m, 1H, Ar**H**), 7.67 – 7.71 (m, 1H, Ar**H**), 8.46 (d, J = 8.0 Hz, 2H, Ar**H**), 12.53 (s, 1H, N**H**); ^{13}C-NMR (DMSO-d$_6$, 100 MHz): δ 114.2, 121.5, 129.7, 130.5, 134.8, 140.9, 152.8; [Anal. Calcd. for $C_{13}H_9BrN_2$: C, 57.17; H, 3.32; N, 10.26. Found: C, 56.79; H, 3.19; N, 9.87], ESI: $[M+H]^+$ m/z 274.13

2-(4-nitrophenyl)-1H-benzo[d]imidazole 3

Benzene-1,2-diamine (540 mg, 5.0 mmol) and 4-nitroaldehyde (755 mg, 5.0 mmol) in methanol (15mL) were reacted according to **GP**. The product was purified by column chromatography on silica gel (50 % EtOAc in hexane) yielded **3** (1.13 g, 4.72 mol, 94.53%); m.p. = 265 ^{o}C IR (KBr): 3351, 3057, 2922, 1619, 1493, 1282 cm^{-1}; ^{1}H-NMR (DMSO-d$_6$, 400 MHz) δ 7.25 (d, J = 7.6 Hz, 2H, Ar**H**), 7.33–7.37 (m, 2H, Ar**H**), 7.41–7.48 (m, 1H, Ar**H**), 7.59–7.64 (m, 1H, Ar**H**), 8.61 (d, J = 7.6 Hz, 2H, Ar**H**), 12.28 (s, 1H, N**H** of Indole); ^{13}C-NMR (DMSO-d$_6$, 100 MHz): δ 115.7, 121.8, 124.4, 137.9, 136.2, 141.1, 147.9, 150.2; [Anal. Calcd. for $C_{13}H_9N_3O_2$: C, 65.27; H, 3.79; N, 17.56; Found: C, 65.9; H, 3.63; N, 1747], ESI: $[M+H]^+$ m/z 712.22

Crystal structure determination

The colourless elongated (in the crystallographic [001] axis direction) prism shaped crystal of compound **1** was sealed in glass capillary filled with helium and next it was mounted on a KM-4-CCD automatic diffractometer equipped with CCD detector, and

used for data collection. X-ray intensity data were collected with graphite monochromated CuK_α (λ = 1.54178 Å) radiation at temperature 100(1) K and in ω scan mode. The 11 seconds exposure time was used, and reflections inside Ewald sphere were collected up to θ= 72.3°. The unit cell parameters were determined from 769 strongest reflections. Details concerning crystal data and refinement are given in Table 1. Examination of reflections on two reference frames monitored after each 20 frames measured showed no loss of the intensity during measurement. During the data reduction Lorentz, polarization and numerical absorption [46] corrections were applied. The structure was solved by partial structure expansion procedure. All the non-hydrogen atoms were refined anisotropically using full-matrix, least-squares technique on F^2. All the hydrogen atoms were found from difference Fourier synthesis after four cycles of anisotropic refinement, and refined as "riding" on the adjacent atom with geometric idealisation after each cycle of refinement and with individual isotropic displacement factors equal 1.2 times the value of equivalent displacement factor of the parent atoms. The SHELXS97, SHELXL97 and SHELXTL [47] programs were used for all the calculations. Atomic scattering factors were taken from International Tables for Crystallography [48]. Selected interatomic bond distances and angles are listed in Table 2 and intermolecular interactions are listed in Table 3.

DNA binding analysis of compound 1, 2 and 3

DNA binding experiments were performed utilizing fluorescence spectrofluorometry. The purity of calf thymus DNA was confirmed by taking the ratio of the absorbance values at 260 and 280 nm in tris-EDTA (10 mM, pH 7.0) buffer, which was found to be 1.8 : 1, indicating that the DNA was sufficiently free of protein and other contaminants.

For measurements all the three compounds were dissolved in 2% DMSO. DNA binding assays of synthesized compounds were carried out in the presence of ethidium bromide. DNA and ethidium bromide (EtBr) were dissolved in tris-EDTA (10 mM, pH 7.0) buffer at concentrations of 4 and 1 μg ml^{-1}, respectively. The concentration of organic compounds 1, 2 and 3 were 50 μM. To perform the experiments, DNA was pretreated with EtBr for 30 min. Then the test solutions were added to this mixture of EtBr–DNA, and the change in the fluorescence intensity was measured. The fluorescence was recorded at 485–685 nm after exciting the solution at 478 nm. The slits were set at 5 nm for excitation and emission. The path length of the sample was 1 cm. To determine the DNA-binding ability of compounds, fluorescence intensity data were analyzed by the Stern–Volmer equation [49].

$$F_0/F = 1+ K_{sv}[Q]$$

Where, F and F_0 are the fluorescence intensity with and without the quencher (complex–DNA), K_{sv} is the Stern–Volmer quenching constant, and [Q] is the concentration of the quencher.

Method for determination of in-vitro cytotoxicity

All compounds tested were dissolved in DMSO and their stock solution of 2 x 10^{-2} M was prepared. Stock solutions were further diluted with complete growth medium supplemented with 50 μg/ml gentamycin to obtain test concentration. Various stock solutions of positive and negative controls were prepared (viz: Adriamycin and paclitaxel in DMSO = 2 x 10^{-3} M; 5-Flurouracil and Mitomycin-C in double distilled water = 2 x 10^{-3}). All the cells were maintained in RPMI-1640 medium and DMEM media,

supplemented with fetal bovine serum (10%), 100 units/ml penicillin and 100 mg/ml streptomycin (complete medium). Viability of cells was evaluated by trypan blue exclusion method immediately before setting up the experiment for cytotoxicity determination. Cells with >98% viability were used in the assay [50]. Briefly, the cells were seeded into 96 well cell culture plates (1 x 10^4 cells/100 µl/well) and incubated in CO_2 incubator (37^0C, 5% CO_2, 95% relative humidity) for 24 h. After 24 h, compounds and positive controls (100 µl/well) were added in quadruplets and the plates were further incubated in CO_2 incubator for 48 h. Suitable controls were also included in each experiment. After 48 h chilled trichloro acetic acid (50% w/v, 50 ml) was laid gently on top of the medium in all the wells. The plates were incubated at 4 ^{o}C for one hour to fix the cells. All the contents of the wells were gently pipetted out and discarded. The plates were washed five times with distilled water to remove trichloroacetic acid, growth medium, low molecular weight metabolites and serum proteins. Further, the plates were air-dried and sulphorhodamine-B (0.4% SRB in 1% acetic acid, 100 µl/well) was added to each well of the 96 well plates and incubated for 30 min. Excess of the dye was washed off using 1% acetic acid and the plates were air-dried. Tris-buffer (10 mM, pH 10.5, 100 ml/well) was added to each well and plates were shaken on a mechanical stirrer for 10 min and O.D. was recorded on ELISA reader at 540 nm. Cell growth in control wells was considered 100% and in turn percent growth inhibition was calculated.

Results and Discussion

*Crystal Structure of compound **1***

A perspective view of compound **1** is shown in Figure 1S. The compound shows minor signs of disorder at terminal atoms, what exhibits in slightly prolated anisotropic displacement ellipsoids of C10, C11 and C12 atoms. All atoms of the compound **1**

occupy the general positions. The 1H-benzimidazole moiety is close to planarity (the maximum deviation from the weighted least squares plane calculated thought all respect non-hydrogen atoms exists for C1 atom and it is equal to 0.022(1) Å), and the planar in the rage of experimental error fused benzene and imidazole rings are inclined at 1.71(9)°. The dihedral angle between the weighted least squares planes of 1H-benzimidazole and chlorophenyl moieties is equal to 39.85(5)°, and it is different from the typical one existing between substituted, above mentioned, moieties (most populated angle values is 5° and over of 50% of observed angles falls in the range 0-15°) [51]. On the basis of bond lengths analysis it can be stated that bonds within the imidazloe ring (Table 2) show rather delocalised character (mean bond length of typical delocalised N-C bond is 1.336 Å [48]) than single or double character (mean bond length of 1.469 in C-NH systems and 1.279 Å in C_{ar}-C=N-C bond system [48], respectively). The C1-C8 bond (linking the rings systems) also shows slight shortening (about 0.05 Å) in comparison to purely single C-C bond. The compound **1** forms one intramolecular and one intermolecular hydrogen bond (Table 3), which, on the basis of system geometry, can be classified as medium strength one [52]. These interactions create the S(6) and C(4) unitary graph set motifs of the lowest degree, and the chain extends along crystallographic [010] axis. Additionally in the structure can be found the two C—H•••π interactions (Table 3) expanding the above mentioned chains to the supramolecular layer propagating along crystallographic (001) plane. The possibility of the existence of a π•••π interactions was rejected on the basis of the long dissonance between centroids of the neighbouring delocalised rings (the shortest one is 4.638(2) Å)

DNA Binding property of Benzimadazole compounds 1, 2 and 3

The fluorescence spectroscopy provides insight of the changes taken place in the microenvironment of DNA molecule on ligand binding. The binding of these compounds with calf thymus DNA was studied by monitoring the changes in the intrinsic fluorescence of these compounds at varying concentration of compounds. Figure 1 shows the representative fluorescence emission spectra of the EtBr-DNA compound. Ethidium bromide, a polycyclic aromatic dye, is the most widely used fluorescence probe for DNA structure. EtBr displays very weak fluorescence in aqueous solution. However, in the presence of DNA, it exhibits intense fluorescence because of intercalation with base pairs in DNA [53]. The gradual decrease in the fluorescence emission intensity of EtBr-DNA complex with a conspicuous change in the emission signals occur due to addition of compounds. The quenching of the compound fluorescence clearly indicated that the binding of DNA to compounds changed the microenvironment of fluorophore residue. The fluorescence data revealed the maximum quenching in compound 2 as compared to 1 and 3. The K_{SV} value of the compound 1, 2 and 3 were calculated to be 0.8×10^4, 1.9×10^4 and 2×10^3 M^{-1}, respectively. A higher K_{SV} value of compound 2 suggests its stronger quenching ability than compound 1 and 2 [Fig 2]

In vitro cyctotoxic assay of compounds 1, 2 and 3

In vitro cytotoxic studies of the compounds were carried out on different cancer cell lines according to the protocol of Skehan et al. [54]. Briefly, the cytotoxic effects were observed on breast (MCF-7), leukemia (THP-1), prostrate (PC-3) and lungs (A-549) cancer cell lines. The cytotoxic effects are reported in terms of percent growth inhibition (Table 4) and IC_{50} values (Table 5). All tested compounds showed concentration

dependent cytotoxicity against all the four cell lines used for the study. Compound **3** was least effective and maximum growth inhibition of 51% was observed at the highest concentration of 100 µM of compound. Compound **1** and **2** showed better growth inhibitory activity as compared to 3. Compound **1,** showed 72-88% and 82-95% growth inhibition at 50 µM and 100 µM respectively. In comparison, Compound **2** showed 68-93% and 71-96% growth inhibition at 50 µM and 100 µM respectively. Interestingly, Compound **2** also showed 66% growth inhibition at 10 µM. Thus, the present study indicates that compounds **1** and **2** have better anticancer activity as compared to compound **3**. Compounds **1** and **2** are more or less equally active but still compound **2** is marginally better than compound **1** in its anticancer activity.

IC_{50} values are well coordinated with the observed values for percent growth inhibition. Compound 2 showed lower IC_{50} values (15µM) as compared to compound 3 and 1, indicating compound 2 have higher affinity against cancer cell lines and more potent anti-cancer agent in comparison to compound 3 and 1 (Table 5). The difference in their activity is due to their different chemical structure.

Supplementary Data

Tables of crystal data and structure refinement, anisotropic displacement coefficients, atomic coordinates and equivalent isotropic displacement parameters for non-hydrogen atoms, H-atom coordinates and isotropic displacement parameters, bond lengths and interbond angles have been deposited with the Cambridge Crystallographic Data Centre under No. CCDC960494.

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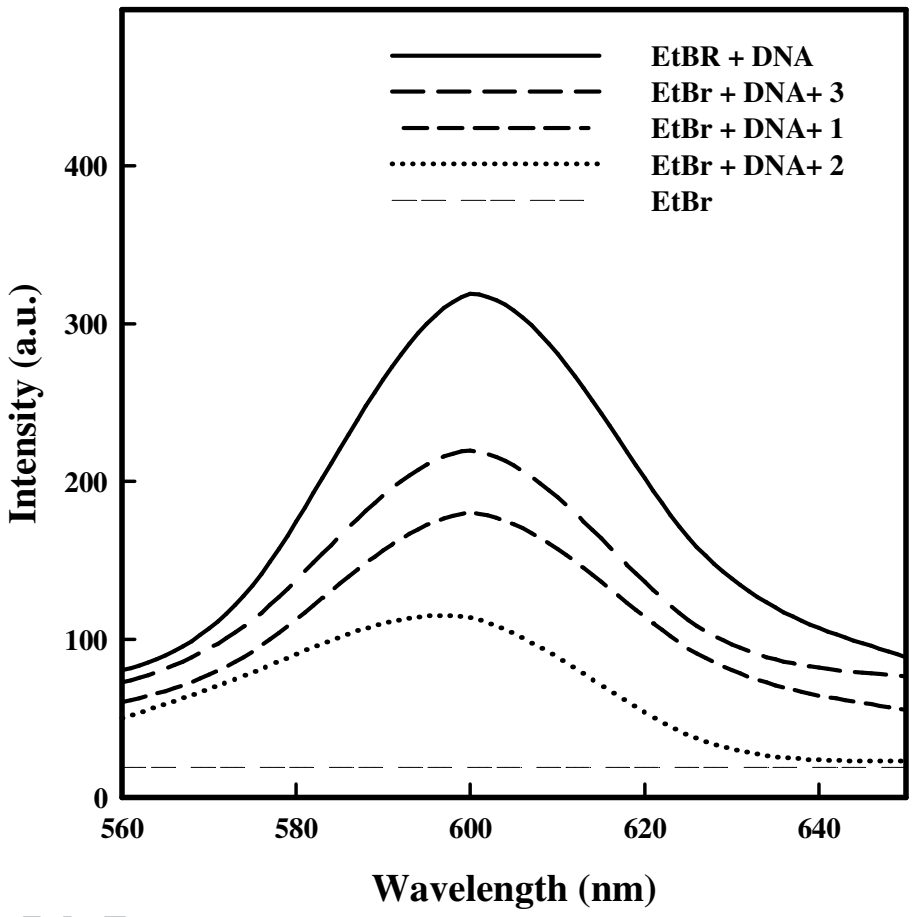
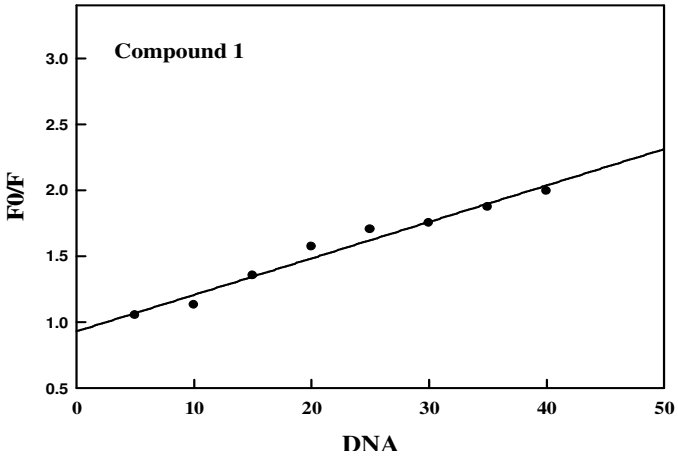
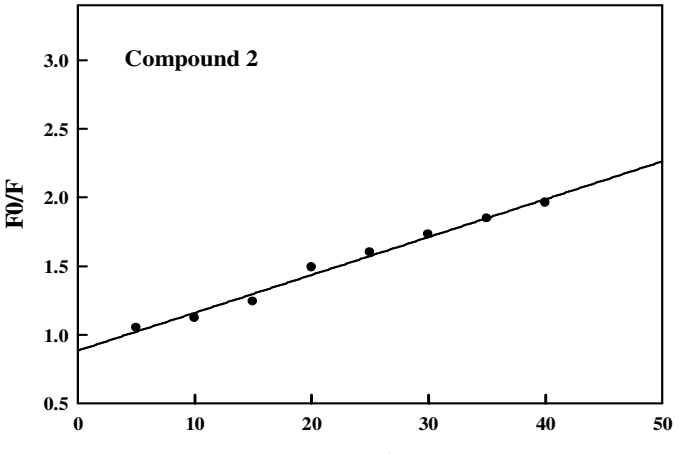


Figure 1: Fluorescence emission spectra of ethidium bromide (EB) bound to DNA in the absence and presence of compound 1, 2 and 3.





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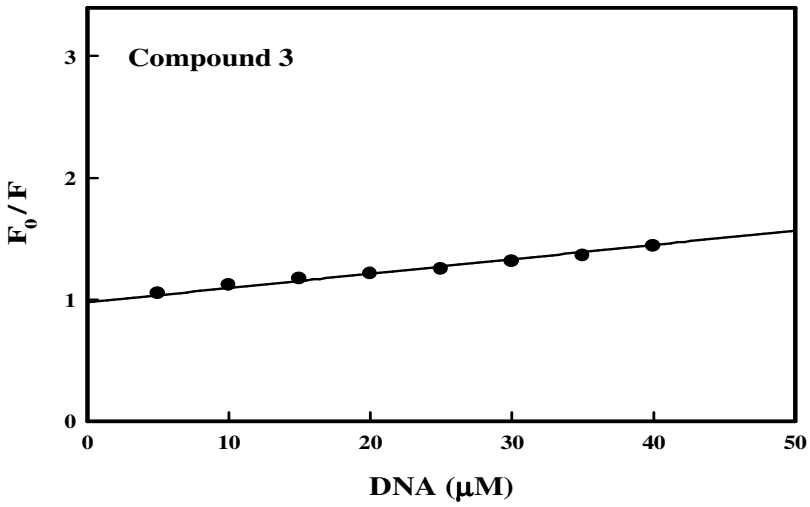


(c)

Figure 2: Stern-Volmer plot of compound 1, 2 and 3 fluorescence quenching

Table 1. Crystal and structure refinement data of the compound **1**

Compound	**1**
Empirical formula	$C_{13}H_9ClN_2$
Formula weight	228.67
Crystal system, space group	orthorhombic, *Pbca* (No. 61)
Unit cell dimensions [Å, °]	a = 7.0197(1)
	b = 9.9261(1)
	c = 31.8686(4)
Volume [Å^3]	2220.55(5)
Z, Calculated density [Mg/m^3]	8, 1.368
Absorption coefficient [mm^{-1}]	2.796
F(000)	944
Crystal size [mm]	0.22, 0.17, 0.08
θ range for data collection [°]	2.77 to 72.30
Index ranges	$-8 \le h \le 8$, $-12 \le k \le 11$, $-39 \le l \le 38$
Reflections collected / unique	23186 / 2194 [$R_{(int)}$ = 0.0246]
Completeness [%]	100.0 (to θ = 67°)
Min. and max. transmission	0.607 and 0.795
Data / restraints / parameters	2194 / 0 / 145
Goodness-of-fit on F^2	1.064
Final R indices [$I>2\sigma(I)$]	$R1$ = 0.0368, $wR2$ = 0.0923
R indices (all data)	$R1$ = 0.0369, $wR2$ = 0.0923
Largest diff. peak and hole [e•Å^{-3}]	0.300, -0.448

Table 2. Selected structural data of the compound **1** [Å, °].

N1—C1	1.3584(17)
N1—C2	1.3764(17)
N2—C1	1.3252(17)
N2—C3	1.3855(17)
C1—C8	1.4752(18)
C1—N1—C2	106.97(10)
N2—C1—N1	112.87(11)
C1—N2—C3	105.05(11)
N1—C1—C8—C9	-41.2(2).
N2—C1—C8—C9	144.37(14)
N1—C1—C8—C13	138.28(14)
N2—C1—C8—C13	-36.18(19)

Table 3. Hydrogen bonds geometry of the compound **1** [Å, °]. Cg1, Cg2 – the centroids of the ring composed from the C1/C2/C3/N1/N2 and C2/C3/C4/C5/C6/C7 atoms, respectively.

D—H•••A	d(D-H)	d(H•••A)	d(D•••A)	<(DHA)
N1—H1N•••Cl1	0.88	2.77	3.0940(11)	103.1
N1—H1N•••N2^{i}	0.88	1.95	2.8006(15)	162.9
C6—H6•••Cg2^{ii}	0.95	2.88	3.6387(16)	138.1
C12—H12•••Cg1^{iii}	0.95	2.97	3.5574(16)	121.6

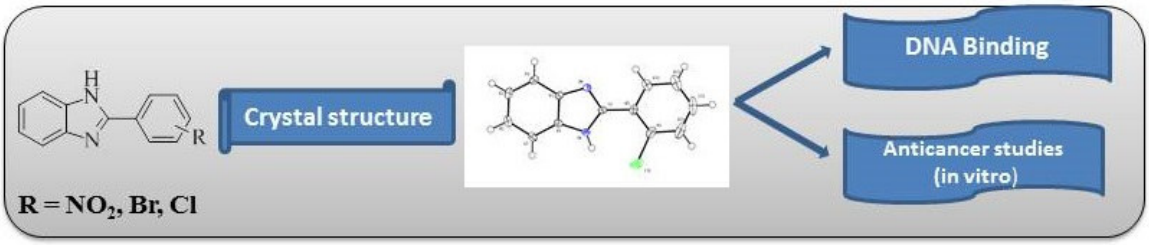
Symmetry transformations used to generate equivalent atoms: (i) -x+1/2, y+1/2, z; (ii) x+1/2, y, -z+1/2; (iii) x-1, y, z.

Table 4: Percent Growth Inhibitory rates of free ligand, L and complexes **1** and **2** against cancer cell line

Tissue			Breast	Leukemia	Prostate	Lung
Cell line type			MCF-7	THP-1	PC-3	A-549
CCL Codes	Inst. Codes	Conc. (µM)	% Growth Inhibition			
M-4460	3	10	38	25	19	15
		50	41	33	28	35
		100	51	55	51	55
M-4461	**1**	10	37	11	32	36
		50	**72**	**88**	**73**	**73**
		100	**94**	**95**	**82**	**85**
M-4462	**2**	10	36	39	**42**	30
		50	**93**	**68**	**60**	**70**
		100	**96**	**71**	**81**	**89**
	Adriamycin	1	72	-	-	-
	Paclitaxel	1	-	-	-	65
	Mitomycin	1	-	-	61	-
	5-Fluorouracil	20	-	67	-	-

Table 5: IC_{50} values of synthesized compounds on various cell lines

	IC_{50} (μM) Cell lines			
Compound	MCF-7	THP-1	PC-3	A-549
1	38 ± 2	43 ± 3	45 ± 1	44 ± 1
2	35 ± 2	48 ± 2	46 ± 1	43 ± 2
3	85 ± 1	86 ± 2	96 ± 1	84 ± 1



1) Synthesis of benzimidazoles derivatives
2) X-Ray crystal structure
3) DNA binding studies
4) Anticancer activities