Articles

Influence of Phenyl Ring Disubstitution on Bisbenzimidazole and Terbenzimidazole Cytotoxicity: Synthesis and Biological Evaluation as Radioprotectors

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DNA minor groove binders, Hoechst 33258 and Hoechst 33342, have been reported to protect against radiation-induced DNA-strand breakage, but their mutagenicity and cytotoxicity limit their use as protectors of normal tissue during radiotherapy and as biological radioprotectors during accidental radiation exposure. On the basis of these observations, two new nontoxic disubstituted benzimidazoles were synthesized, one having two methoxy groups (5-(4-methylpiperazin-1-yl)-2-[2′-(3,4-dimethoxyphenyl)-5′-benzimidazolyl]benzimidazole, **5**) and another having a methoxy and a hydroxyl group (5-(4-methylpiperazin-1-yl)-2-[2′{2″-(4-hydroxy-3-methoxyphenyl)-5″-benzimidazolyl}-5′-benzimidazolyl]benzimidazole, **6**) ortho to each other on the phenyl ring. The radiomodifying effects of these nontoxic ligands were investigated with a human glioma cell line exposed to low linear energy transfer radiation by determining cell survival and cell proliferation compared with effects of the parent compound, Hoechst 33342. Cytotoxicity assayed by analyzing clonogenicity, cell growth, and metabolic viability showed that both **5** and **6** were nontoxic at 100 μM after 72 h of exposure, whereas Hoechst 33342 resulted in lysis of 77% of these cells in 24 h. Macrocolony assay (clonogenicity) showed that 73%, 92%, and 10% of the cells survived when treated with 100 μM **5**, **6**, and Hoechst 33342, respectively. Both **5** and **6** did not affect the growth of BMG-1 cells. At 10 μM, **5** and **6** showed 82% and 37% protection against radiation-induced cell death (macrocolony assay) while 100% protection was observed against growth inhibition. Disubstitution of the phenyl ring has not only reduced cytotoxicity but also enhanced DNA-ligand stability, conferring high degree of radioprotection.

Introduction

DNA ligands such as bisbenzimidazoles Hoechst 33258 and Hoechst 33342 form strong and noncovalent bonds with the adenine- and thymine-rich regions in the minor groove of DNA, significantly altering chromatin structure.[1,2] Dyes Hoechst 33258 and Hoechst 33342 are frequently used in cytometry to stain chromosomal materials in situ because these two compounds become highly fluorescent upon bonding to DNA.[3] It has been known for a long time that Hoechst 33258 bonds specifically to AT-rich sequences in DNA.[4−8] NMR and X-ray crystal structures of Hoechst 33258 bound to different oligonucleotide duplexes reveal that the drug fits into the minor groove of the double helix for a sequence of four contiguous AT base pairs.[9−17] The Hoechst 33258−DNA interaction appears to be stabilized by several H-bonding and van der Waals bonding interactions,[18] and these interactions are suggested to contribute to overall bonding affinity.[19] The hydrophobic interaction with DNA bonding sites is more likely to represent the main driving force for complex formation.[20] Synthesis of a number of bisbenzimidazoles equipped with various side chains have been reported.[21−26] The central part of the parent bisbenzimidazole has also been modified with the aim of designing compounds capable of recognizing GC containing sequences.[27,28] Recently several bisbenzimidazoles and terbenzimidazoles have been identified as topoisomerase I inhibitors.[29−39]

Hoechst 33258 and also Hoechst 33342 have been shown to protect nonbonded as well as genomic DNA against radiation-induced damage.[40] Administration of these compounds prior to irradiation affords protection against the formation of primary strand breaks in aqueous solutions of DNA as well as in the intact cell nucleus. These DNA-ligand interactions have also been observed to reduce radiation-induced cytogenetic damage and cell death in cell cultures and to protect whole body irradiated animals.[41−43] However, postirradiation

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treatment of cells with these ligands has been observed to enhance cell death in vitro.[44] Free radical scavenging and quenching of DNA radicals appear to be the mechanisms responsible for the protection of DNA against radiation-induced damage by Hoechst compounds,[44−47] while mechanisms involved in the enhancement of radiation-induced cell death, when administered after irradiation, are not clearly understood.

The mutagenic, clastogenic, and cytotoxic effects of these ligands arising from DNA lesions caused by topoisomerase inhibition, resulting in altered gene expression and DNA repair inhibition, limit their use as radioprotectors in humans. Therefore, the development of DNA bonding ligands (minor groove binding ligands particularly) that afford a radioprotective effect without causing mutagenic and cytotoxic effects can play a significant role in biological radioprotection.

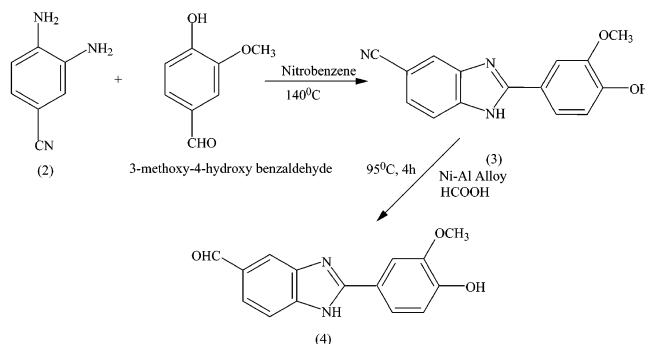
In the present study, detailed synthesis of two benzimidazoles 5-(4-methylpiperazin-1-yl)-2-[2′-(3,4-dimethoxyphenyl)-5′-benzimidazolyl]benzimidazole **5** and 5-(4-methylpiperazin-1-yl)-2-[2′{2″-(4-hydroxy-3-methoxyphenyl)-5″benzimidazolyl}-5′-benzimidazolyl]benzimidazole **6** and the relative structure−activity of 3,4-dimethoxybisbenzimidazole and 4-hydroxy-3-methoxyphenylterbenzimidazole as radioprotective agents were compared to those of Hoechst 33342 using a human glioma cell line (BMG-1). Addition of an extra methoxy group to the phenyl ring was expected to significantly reduce cytotoxic effects in vitro.[33,37] The reduced cytotoxicity of these ligands makes them promising candidates as radioprotective agents. Effects on cell survival, macrocolony formation, and growth inhibition have been investigated as parameters relevant to protection against radiation-induced damage. Results indicate that these novel ligands not only demonstrate radioprotective effects but also are not toxic under in vitro conditions.

Chemistry

In general, the synthesis of 2-arylbenzimidazoles have been effected by condensation of *o*-arylenediamine and arylcarboxylic acids and their derivatives ester, amide, imidate esters, chlorides, and anhydrides in the presence of a strong acid such as a mineral acid or polyphosphoric acid. 2-Arylbenzimidazole can also be obtained from *o*-arylenediamines and aldehydes in the presence of an oxidant.

The synthesis of **5** was carried out basically as described by Kelly et al.[48] using 2-amino-4- [5′-(4″-methylpiperazin-1″-yl)benzimidazol-2′-yl]aniline (**1**) and 3,4-dimethoxybenzaldehyde in 27% overall yield. This methodology is different from the method described by Lown et al.[49−51] and Douglas et al.[52] where they have condensed 2-arylbenzimidazole with *o*-arylenediamine. **6** was prepared using a methodology described by La Voie et al.[33] using an equimolar mixture of **1** and 5-formyl-[3-methoxy-4-hydroxybenzimidazole] (**4**) in nitrobenzene that was heated at 140−150 °C for 36 h. An overall yield of 25% was obtained for **6**. In this reaction, the initially formed Schiff base undergoes oxidative cyclization in nitrobenzene to give **6**. **1** was synthesized by condensing ethyl-4-amino-3-nitrobenzenecarboximidate hydrochloride[48] and 5-(4′-methylpiperazin-1′yl)-2-nitroaniline in dry ethanol/glacial acetic acid refluxed for 1 h to give 4-[5′-(4″-methylpiperazin-1″-yl)benzimidazol-2′-yl]-2-nitroaniline, which was hydrogenated over 5% Pd/C to give **1** in 100% yield. **4** was prepared by condensing 3,4-diaminobenzonitrile with 3-methoxy-4-hydroxybenzaldehyde (vanillin), a methodology described by La Voie et al.[34−37] The nitrile group was reduced to a formyl group using a Ni−Al alloy to obtain **4** (Schemes 1 and 2).

Scheme 1

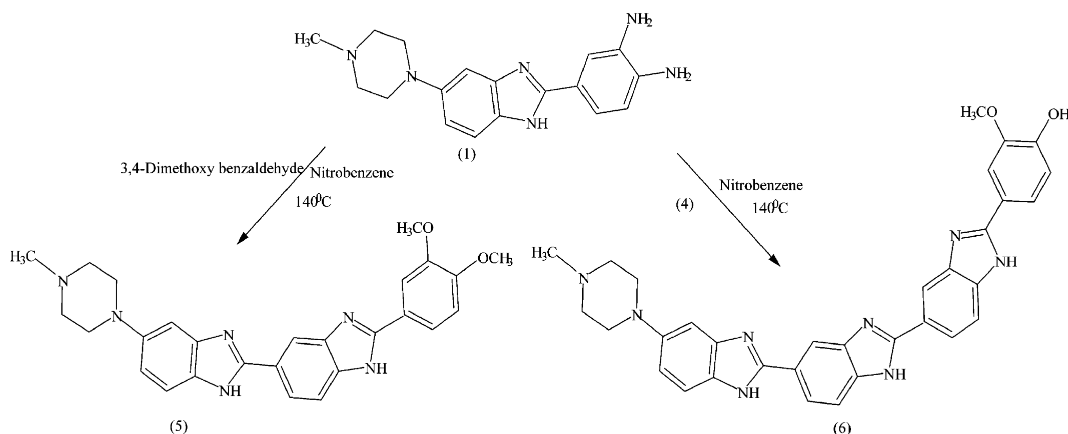


Two chemical modifications were introduced into bisbenzimidazole and terbenzimidazole. For bisbenzimidazole, two methoxy groups were introduced at the phenyl ring, which are electron donors, whereas in the terbenzimidazole, one of the methoxy groups was replaced by a hydroxyl group. These modifications produced increased lipophilicity but reduced cytotoxicity. At the same time, these ligands were found to provide better protection against radiation-induced cytotoxicity than monosubstituted Hoechst analogues.

Results and Discussion

Hoechst 33342 has been shown to be cytotoxic to various types of mammalian cells.[53] The increased cytotoxicity of Hoechst 33342 has been suggested to be due to lipophilicity of the molecule, which increases cell penetration of the drug.[29] Earlier studies have also shown that substitution of the phenyl ring at the 5′-position of terbenzimidazole increased its cytotoxicity toward mammalian cells.[33] Compounds **5** and **6** were different from analogues synthesized by La Voie et al.[34−37] and Leupin et al.[54] because they have a methylpiperazine ring at the 5-position and methoxy and hydroxyl groups at the 3′- and 4′-positions on the phenyl ring. It was suggested that substitution with a methoxy group would increase the lipophilicity, leading to a higher uptake by mammalian cells and resulting in enhanced cytotoxicity. Besides a lipophilicity-linked uptake, the intracellular concentration of the drug that determines its toxicity is also regulated by the P-glycoprotein (Pgp) mediated drug efflux.[55] Therefore, our results suggest that both **5** and **6** are handled by the Pgp pump similar to Hoechst 33342. The lipophilicity of **5** and **6** determined by measuring the uptake of these compounds by lymphocytes isolated from Wistar rat spleen was compared to that of Hoechst 33342 (Figure 1). The uptake of Hoechst 33342, **5**, and **6** as indicated by the fluorescence intensity of live lymphocytes suggests that all three ligands enter cells and bond to nuclear DNA. The difference in fluorescence intensity after 1 h suggests that Hoechst 33342 and **5** are secreted from cells in a similar fashion whereas **6** seems to remain inside the cells for a longer time. It is notable here that the inherent fluorescence of **5** and **6** is less

Scheme 2



than that of Hoechst 33342, but upon bonding to DNA, these two ligands show higher fluorescence in comparison to Hoechst 33342, $R_f = 16$ and $R_f = 100$, respectively.[61] It was observed that addition of a methoxy group increases cellular uptake of these compounds in comparison to Hoechst 33342. The cytotoxicity of these ligands was investigated using a human glioma cell line (BMG-1) by studying their effects on cell survival, metabolic viability, and cell proliferation. For survival and proliferation studies, cells were incubated with **5**, **6**, and Hoechst 33342 for 1 h and washed to remove the drug, and growth was monitored as colony formation. Cell survival was determined following the addition of 0.1, 1, 10, or 100 μM drug. At low concentrations, <1 μM, Hoechst 33342 had no effect on cell survival, whereas at 10 or 100 μM, it killed 55% or 90% of these cells, respectively. On the other hand, **6**, even at 100 μM, did not show any adverse effect on cell survival (Figure 2). Similarly, **5** was also not cytotoxic at concentrations up to 10 μM, while 27% cells were killed with the addition of 100 μM. Effects of **5** and **6** on the proliferation of exponentially growing cells at 100 μM were also studied. In these experiments, cells were incubated with **5**, **6**, or Hoechst 33342 for 1 h, washed to remove the drug, and incubated further for 24, 48, and 72 h to observe growth. Kinetics of cell growth treated with **5** or **6** did not differ significantly from the kinetics of the nontreated cells, and doubling time was comparable to that of the nontreated cells (Figure 3). However, 100 μM Hoechst 33342 induced complete lysis of these cells (Figure 3). Taken together, these results suggest that **5** and **6** are significantly less toxic than Hoechst 33342.

Cytotoxicity was further investigated by oxidation of MTT as described earlier[49,51,56] by incubating cells with drugs continuously. It was observed that when 10 μM Hoechst 33342 was incubated with BMG-1 cells for 72 h, 35% of the cells were killed whereas 100 μM **5** and **6** showed no cytotoxicity. Incubation of cells with 100 μM Hoechst 33342 for 72 h showed complete lysis of cells. Interestingly only a marginal increase in the cytotoxicity

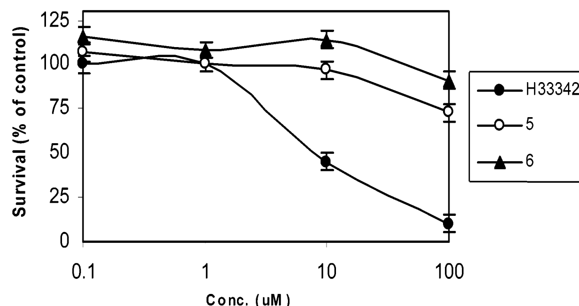


Figure 2. Effect of **5** and **6** on the clonogenicity of BMG-1 cells exposed to various concentrations at 24, 48, and 72 h. Cells were plated and allowed to form colonies in the presence of various concentrations of drugs. Results are shown as the percent of the control. **5** and **6** were found to have no effect on clonogenicity of BMG-1 cells.

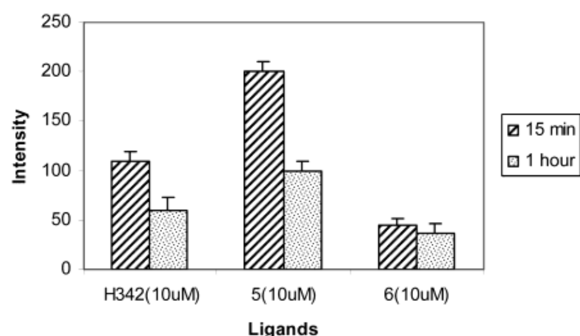


Figure 1. Cellular uptake of 10 μM Hoechst 33342, **5**, and **6** by live lymphocytes isolated from Wistar rat spleen. Cells were incubated with ligands for 15 min and 1 h and dropped on clean chilled glass slides. Results are shown as the mean of the fluorescence intensity for 100 cells in each group. Hoechst 33342 and **5** appear to be secreted by the Pgp mediated pump, whereas **6** seemed to be retained inside these cells.

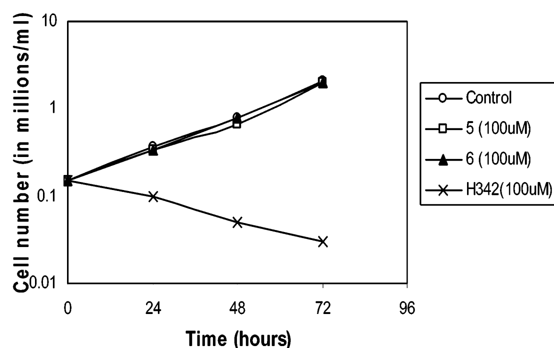


Figure 3. Effect of 100 μM **5** and **6** on growth kinetics of BMG-1 cells. Cells were grown in the presence of these drugs, and the number of proliferating cells were counted using a hemocytometer at different time intervals. Results are shown in terms of the number of cells (in millions per milliliter). **5** and **6** do not show any effect on the proliferation kinetics of BMG-1 cells.

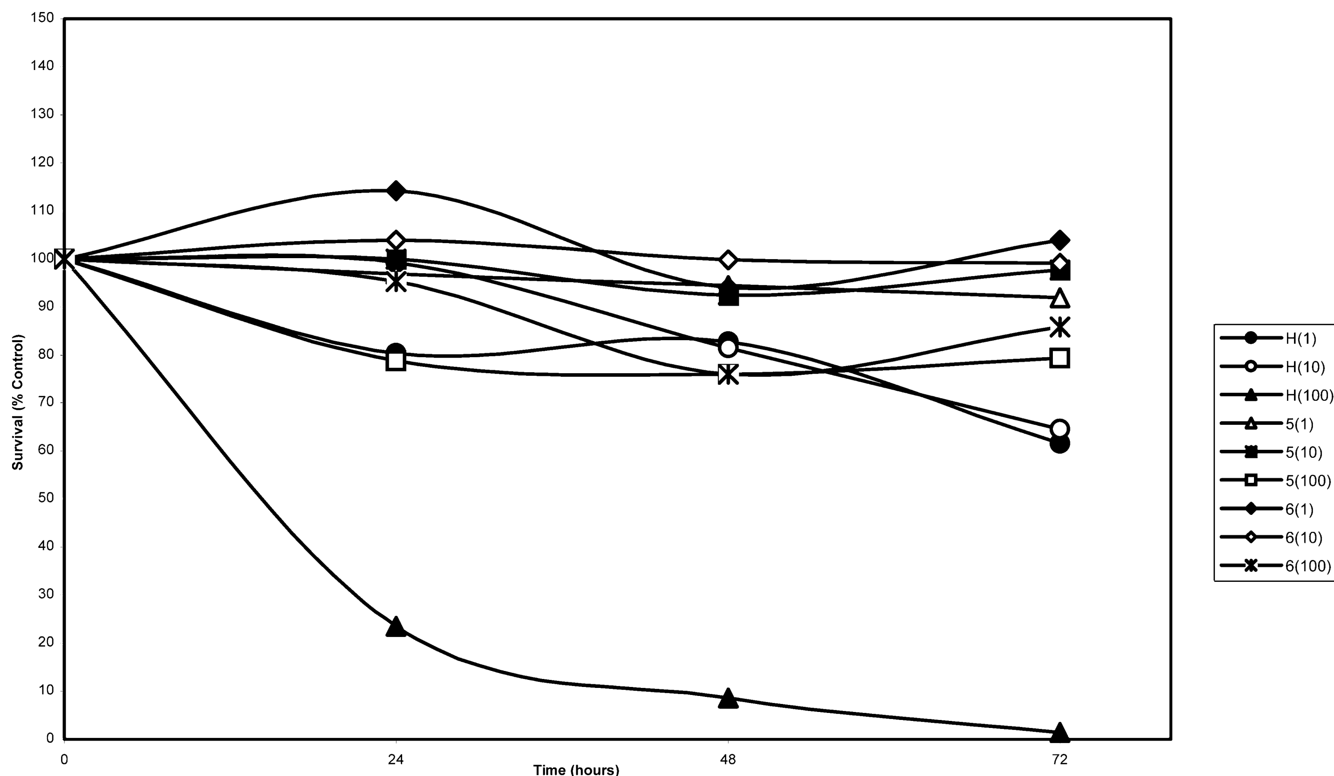


Figure 4. Cytotoxicity of **5** and **6** compared to Hoechst 33342 using the MTT assay of human brain malignant glioma cells, BMG-1. Cells were grown in the presence of drugs at various concentrations up to 72 h, and the metabolic activity of these cells was measured using MTT. Results are shown as the percent survival. **5** and **6** were found to be noncytotoxic even at 100 μM up to 72 h.

was observed with 100 μM **5** and **6** (Figure 4). The cytotoxicity of Hoechst 33342 is primarily due to the stabilization of cleavable complexes, the protein-linked DNA strand breaks, an intermediate process of DNA topoisomerase action.[57] Cleavable complexes have been shown to induce interphase death via apoptosis[58] as well as mitotic death linked to the cytogenetic damage in the form of chromosomal aberrations and micronuclei formation.[59] The absence of cell death with **5** and **6** observed here implies that either the intracellular concentration of these ligands is significantly lower than the cellular concentration of Hoechst 33342 or they do not stabilize the cleavable complexes, unlike Hoechst 33342. However, significant differences in the uptake of these ligands and Hoechst 33342 were not observed. Therefore, it appears that the inability of these ligands to stabilize cleavable complexes is mainly responsible for reduced toxicity.

The DNA bonding property of **5**, **6**, and Hoechst 33342 was determined with calf thymus DNA (CTDNA) by thermal denaturation, absorption, and emission spectroscopy. Incubation of 10 μM drug with 100 μM CTDNA was performed, and thermal denaturation spectra were recorded. Results suggest that **5** stabilizes the duplex, similar to Hoechst 33342, but **6** conferred higher duplex stability (Table 1). In their DNA bonding activity, Hoechst 33342 and **5**, both being bisbenzimidazoles, have similar properties whereas **6**, a terbenzimidazole, binds DNA in a different manner as evident from UV and fluorescence spectrophotometry and thermal denaturation studies. The disubstituted **5** and **6** are more lipophilic and have higher cell permeability compared to Hoechst 33342, which again suggests that cell permeability is not the only factor responsible for increased cytotoxicity of Hoechst 33342 compared to Hoechst 33258.

Table 1. DNA Binding Properties of the Ligands with CTDNA in 2 mM Sodium Cacodylate Buffer (pH 6.9)

study system	λ_{ex} (nm)	λ_{em} (nm)	T_m (°C)	ΔT_m (°C)
CTDNA (100 μM)			70	
CTDNA+ Hoechst 33342 ($r = 0.1$[a])	354	484	76	6
CTDNA + **5** ($r = 0.1$[a])	354	484	76.4	6.4
CTDNA + **6** ($r = 0.1$[a])	356	391, 418, 441	78	8
Hoechst 33342 (10 μM)	340	500		
5 (10 μM)	340	500		
6 (10 μM)	350	500		

[a] r is the ratio of drug to DNA (drug/DNA).

The limitation of the parent Hoechst 33342 compound being mutagenic and, as a result, cytotoxic prevents its use as a radioprotective agent. Since **5** and **6** did not possess these limitations, their potential as radioprotective agents was explored and compared with that of Hoechst 33342. Macrocolony assay was performed following irradiation with one of three radiation doses (2, 5, or 10 Gy) and incubation with medium containing 10 μM ligand (Figure 5). With a radiation dose of 2 Gy, Hoechst 33342 produced 10% protection whereas **5** and **6** produced 24% protection, and with a radiation dose of 5 Gy, Hoechst 33342 produced 15% cell survival, **5** produced 82% cell survival, whereas **6** produced 37% cell survival. Strikingly, with 10 Gy, both **5** and **6** produced 100% protection with respect to irradiated control cells, whereas Hoechst 33342 produced 15% survival. Similar results were obtained for radiation-induced growth inhibition, where 84% protection was observed at 24 h with **5** whereas **6** produced 100%

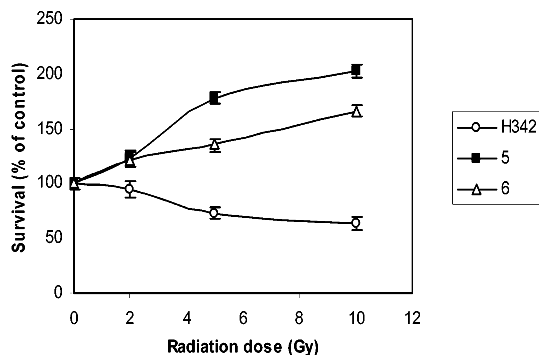


Figure 5. Radioprotection afforded by **5** and **6** compared to Hoechst 33342 studied by macrocolony assay. BMG-1 cells were seeded in Petri dishes and exposed to radiation doses of 2, 5, and 10 Gy at room temperature. These irradiated cells were plated and allowed to form colonies. Results are shown as the percent of control cells irradiated at the respective doses. **5** and **6** are shown to be better radioprotectors compared to Hoechst 33342.

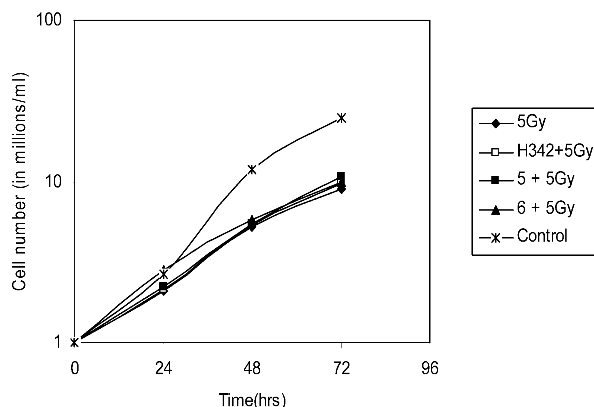


Figure 6. Radioprotective effect of **5** and **6** determined as growth kinetics of BMG-1 cells after exposure to a 5 Gy radiation dose. Cells were irradiated after treatment with a ligand and then counted using a hemocytometer at 24, 48, and 72 h. Results are shown as cell number (in millions per milliliter). BMG-1 cells showed similar proliferation kinetics in the presence of **5** and **6** as control, whereas Hoechst 33342 caused cell killing after irradiation.

protection (Figure 6). Bonding of these ligands to the DNA is characterized by a blue shift in emission and an increase in fluorescence intensity that is suggested to be due to the displacement of water molecules from the spine of hydration in DNA.[12,13] Displacement of water molecules minimizes free radical generation upon radiolysis of water due to ionizing radiation, which is a primary cause of radiation-induced damage. Both **5** and **6** produced high relative fluorescence enhancement upon bonding to DNA (R_f = 16 and R_f = 100, respectively),[61] displacing the spine of hydration from the DNA, and thus showed enhanced protection against radiation compared to Hoechst 33342. The majority of strand breaks in irradiated DNA may be produced by radical species formed near the nucleic acid bases, which subsequently abstract H atoms from deoxyribosyl nucleic acid bases. Delocalization of the radical center on these bases could involve the O2 of pyrimidines, and it is clear that H atom donation is possible from these ligands. The proximity of H atom donors (viz. benzimidazole NHs) on the ligand and H-bond acceptors (viz. thymine O2s and adenine N3s) on the DNA contributes to the stability and selectivity of these complexes.[6,19] **6** has a longer structure and is able to recognize more base pairs that are evident through the relative fluorescence enhancement and T_m studies. The higher bonding affinity but noncytotoxicity of **6** is suggested to be due to the cavities that remain after the bonding of **6** to DNA that allow the religation as well as rotation of single-stranded DNA to facilitate DNA replication. Since metal chelation has been convincingly demonstrated to reduce the radiation damage in a number of systems,[60] the possibilities of minor groove binding ligands to facilitate this process is worth investigating.

Conclusion

5 and **6** have electron-donating groups on the phenyl ring compared to Hoechst 33342; thus, the complex formed by these ligands with DNA have enhanced stability and also selectivity, which may prevent the formation of free radical species on these bases. These ligands also scavenge the free radicals being generated in the vicinity and thereby afford better protection against radiation-induced DNA damage. Hence, it can be concluded that disubstitution of electron-donating groups on the phenyl ring of **5** and **6** enhances cellular uptake but reduces cytotoxicity and affords better radioprotection.

Experimental Section

Chemicals. Chemicals and reagents used in the present studies were analytical reagent grade or equivalent and were procured from Sigma Chemical Co., E. Merck (Germany), Fine Chemicals (India), and Fluka Chemicals. Organic Solvents were freshly distilled before use. The purity and homogeneity of all organic compounds were confirmed using NMR and reverse-phase HPLC. IR spectra were recorded with an FTIR-8300 Shimadzu spectrophotometer. NMR spectral characterization of all synthetic compounds were performed with a Bruker 300 MHz NMR instrument. The FAB mass spectra were recorded on a JEOL SX 102/DA-6000 mass spectrometer using *m*-nitrobenzyl alcohol (NBA) as the matrix. Mass spectra of compound **6** were obtained with a MALDI Kratos Analytical Kompact SEQ IV, U.K., mass spectrometer using α-cyano-4-hydroxycinnamic acid as a matrix.

General Procedure. The synthesis of 4-[5′-(4″-methylpiperazin-1″-yl)benzimidazol-2′-yl]-2-nitroaniline and ethyl-4-amino-3-nitrobenzenecarboximidate hydrochloride were performed according to the procedures described by Kelly et al.[48] and Lown et al.[26−28]

2-Amino-4-[5′-(4″-methylpiperazin-1″-yl)benzimidazol-2′-yl]aniline (1). A solution of 4-[5′-(4″-methylpiperazin-1″-yl)benzimidazol-2′-yl]-2-nitroaniline (1.04 g, 30 mmol) in ethyl acetate/methanol (80 mL of 2:1 mixture) was reduced with 5% palladium on carbon (250 mg) at room temperature and atmospheric pressure. When hydrogen uptake ceased, the solution was filtered through a Celite bed and the filtrate was concentrated without delay to afford the orange-brown diamine **1** in 100% yield.

3,4-Diaminobenzonitrile (2). A solution of 4-aminobenzonitrile (1.5 g, 9.4 mmol) in ethyl acetate/methanol (80:20) was reduced over 10% Pd/C at room temperature and atmospheric pressure. After the hydrogen uptake ceased, the solution was filtered with the aid of Celite and the filtrate was concentrated to dryness to obtain the desired diamine **2** in 100% yield.

5-Cyano-2-[3-methoxy-4-hydroxybenzimidazole]-5′-yl (3). A solution of 1.224 g (9.2 mmol) of the diamine **2** and 1.4 g (9.2 mmol) of 3-methoxy-4-hydroxybenzaldehyde in nitrobenzene was allowed to react in a three-necked round-bottom flask under nitrogen at 140 °C. The reaction mixture was heated for 18 h with stirring and nitrobenzene was then removed

under reduced pressure to obtain the brown crude product **3**. The final product was then obtained using silica gel (60−120 mesh size) column chromatography with EtOAc/MeOH as eluent: mp 226−230 °C; IR 3425.3 (O−H), 3263.3 (−NH), 2221.8 (−CN), 1278.7 (C−O−C) cm^{-1}; ^{1}H NMR δ (in ppm) 13.5 (b, 1H, −NH), 9.52 (s, 1H, −OH), 7.85 (d, 1H, C7), 7.82 (d, 1H, C6), 7.72 (s, 1H, C3′), 7.63 (d, 1H,C5′), 6.93 (d, 1H, C6′), 3.90 (s, 3H, $-OCH_3$).

5-Formyl-[3-methoxy-4-hydroxybenzimidazole] (4). To a solution of 1 g (3.77 mmol) (3) in 60 mL formic acid and 20 mL of water, Ni−Al alloy (3.96 g) was added in several portions. The reaction mixture was heated at 95 °C for 4 h. The hot mixture was filtered with the aid of Celite and washed thrice with water. The solution was concentrated to dryness and water added to the residue to obtain a white precipitate. The pH of this suspension was adjusted to 9 by the dropwise addition of 2 N NaOH. The product was obtained by extraction with ethyl acetate. The ethyl acetate extract was dried over Na_2SO_4 and concentrated in vacuo to give the yellow colored compound in 50% yield: mp 260−264 °C; IR 3435.6 (−OH), 3194.55 (−NH), 1675.6 (−CHO), 1594, 1506, 1441.6, 1280.7 cm $^{-1}$; ^{1}H NMR δ (in ppm) 13.7 (b, 1H, −NH), 10.2 (s, 1H, −CHO), 9.5 (s, 1H, −OH), 8.25 (d,1H, C6), 7.78 (s,1H, C4), 7.75 (d,1H, C7), 7.7 (s, 1H, C3′), 7.65 (d,1H, C5′), 6.92 (d,1H, C6′), 3.92 (s,3H, $-OCH_3$).

5-(4-Methylpiperazin-1-yl)-2-[2′-(3,4-dimethoxyphenyl)-5′-benzimidazolyl]benzimidazole (5). A solution of freshly prepared diamine **1** (1.18 g, 3.67 mmol) and 3,4-dimethoxybenzaldehyde (0.61 g, 3.67 mmol) in 110 mL of nitrobenzene is heated at 140−150 °C for 24 h. The solvent is then removed under reduced pressure to give the final crude product as a brown solid. The product is purified by column chromatography on a Buchi 688 liquid (MPLC) pump using silica gel (70−230 mesh size) and EtOAc/MeOH as eluent to give a yellow compound that was characterized by spectroscopic techniques: mp 220 °C; yield 60%; IR 3556, 2922, 1629, 1508, 1417, 1371, 1296, 1022, 810 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ (in ppm) 2.24 (s, 3H, $-NCH_3$), 2.46 (t, 4H,CH_2, $J = 4$ Hz), 3.5 (t, 4H, CH_2, $J = 4$ Hz), 3.86 (s, 3H,OCH_3), 6.77 (d, 1H, Ar−H, $J = 8$ Hz), 7.13 (d, 2H, Ar−H, $J = 9$ Hz), 7.64 (d, 1H, Ar−H, $J = 8.5$ Hz), 7.78 (d, 1H, Ar−H, $J = 8$ Hz), 8.03 (m, 1H, Ar−H), 8.17 (d, 2H, Ar−H, $J = 9$ Hz), 8.3 (s, 1H, Ar−H), 13.0 (bs, 2H, NH). D_2O exchange resulted in the disappearance of the peak at δ 13.0. EIMS 467 (M^+), 425, 411, 261, 235, 220, 194, 118, 91, 55, 44. $C_{27}H_{28}N_6O_2$: C = 69.23 (calcd), 69.25 (obsd); H = 5.98 (calcd), 5.97 (obsd); N = 17.95 (calcd), 17.93 (obsd).

5-(4-Methylpiperazin-1-yl)-2-[2′{2′′-(4-hydroxy-3-methoxyphenyl)-5′′-benzimidazolyl}-5′-benzimidazolyl]benzimidazole (6). A mixture of freshly prepared diamine **1** (218 mg, 0.82 mmol) and **4** (219 mg, 0.82 mmol) was dissolved in 30 mL of nitrobenzene and heated at 140 °C for 36 h. The addition of **4** was performed as three additions to the reaction mixture to obtain a better yield. Nitrobenzene was removed under reduced pressure and the resulting solid was purified by column chromatography (EtOAc/MeOH) on a Buchi 688 liquid (MPLC) pump using silica gel (70−230 mesh size) to obtain the final product in 54% yield as a brown solid: mp >290 °C; IR 3435, 3195, 1632, 1560, 1413, 1281; ^{1}H NMR δ (in ppm) 13.4−13.55 (b, 3H), 9.5 (s, 1H), 8.45 (s, 1H), 8.39 (s, 1H), 8.03−8.08 (m, 4H), 7.9 (d, 1H), 7.68 (d, 1H), 7.38 (d, 1H), 6.91−6.98 (m, 3H), 3.92 (s, 3H), 3.23 (t, 4H), 2.68 (t, 4H), 2.60 (s, 3H). The mass to charge ratio of **6** was observed with MALDI: 570.3. $C_{33}H_{30}N_8O_2$: C = 69.35 (calcd), 69.34 (obsd); H = 5.429 (calcd), 5.43 (obsd); N = 19.62 (calcd), 19.64 (obsd).

Cell Culture. The human glioma cell line BMG-1 (DNA index of 0.96; wild-type TP 53) was established from a human mixed glioma.[62] Cells were maintained as monolayers at 37 °C in 25 cm^2 tissue culture flasks (Tarsons, India) using Dulbecco's modified Eagle's medium (Sigma) supplemented with 5% fetal calf serum (Biologicals, Israel). Cells were passaged routinely in the exponential growth phase twice a week using 0.05% trypsin−EDTA solution in phosphate-buffered saline (PBS) for trypsinization. All experiments were performed with asynchronously growing cells in the exponential growth phase (24 h after plating).

Cytotoxicity Assay. Cytotoxicity was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay[49,51,56] using a 96-well microtiter plate. Three-thousand cells per well were plated in 200 μL of the complete medium, and treatment with these ligands was performed 24 h after plating. For IC_{50} determination, cells were exposed continuously with varying concentrations of drug and MTT assays were performed at the end of the fourth day. At the end of the treatment, control and treated cells were incubated with MTT at a final concentration of 0.5 mg/mL for 2 h at 37 °C and then the medium was removed. The cells were lysed and the formazan crystals were dissolved using 150 μL of DMSO. The absorbance was read at 570 nm using 630 nm as the reference wavelength using an enzyme linked immunosorbent assay (ELISA) reader.

Macrocolony Assay. BMG-1 cells were washed with HBSS, plated, and grown for 4 days before harvesting using 0.05% trypsin. Depending on the treatments, 200−1200 cells were plated in 90 mm Petri dishes and incubated at 37 °C in a 5% CO_2 humidified atmosphere for 8−10 days. Colonies were fixed with methanol and stained with 1% crystal violet. Colonies containing more than 50 cells were counted.

Proliferation Kinetics. BMG-1 cells were seeded at 7000−8000 cells/cm^2, and their proliferation kinetics was studied at 24 h intervals following trypsinization and counting total cells per flask using a hemocytometer.

Irradiation Procedures. Exponentially growing cells, 24 h after plating, were irradiated at room temperature in growth medium with a Co-60 source (γ cell, AECL, Canada) at a dose rate of 0.5−1.2 Gy/min. A concentration of 10 μM drug was added 1 h before irradiation. Following this procedure, cells were grown for various time intervals to study growth kinetics and plated for macrocolony assay.

Drug−DNA Binding Studies. CTDNA was purchased from E. Merck, Germany, and used without further purification. Absorbance of **5** ($\epsilon_{340} = 20\,300$) and **6** ($\epsilon_{354} = 16\,600$) was compared with that of Hoechst 33342 in sodium cacodylate buffer (2 mM, pH 7.2), and the absorbance spectra were recorded with a UV−visible GBC-916 spectrophotometer (Australia). Drug−DNA solutions (drug/DNA ratio $r = 0.1$) were prepared in sodium cacodylate buffer (2 mM, pH 7.2). Solutions of drug and DNA were incubated for 25 min for equilibration. The spectral measurements were recorded using a 1 cm path length quartz cell in the range 200−500 nm. Fluorescence measurements were made with a custom-designed integrated steady-state and time-resolved spectrofluorimeter model FS900/FL900CDT Edinburgh Analytical Instruments, U.K. The instrument parameters were as follows: $\lambda_{ex} = 340$ nm (for drugs) and $\lambda_{ex} = 350$ nm (for complex), slit width = 10 mm, EHT = 6.7, gap = 0.8 mm, frequency = 40 kHz. Data acquisition was based a on time-correlated single-photon counting (TCSPC) technique. Melting temperature (T_m) measurements were performed with a Cary UV−vis spectrophotometer using 1 cm path length quartz cells equipped with a thermoprogrammer. Absorbance was monitored at 260 nm, while the temperature was raised from 25 to 100 °C at the rate of 0.5 °C/min. The transition melting temperature, T_m, was determined as the midpoint of the normalized curves.

Cellular Uptake by Lymphocytes. Lymphocytes were isolated from Wistar rat spleen as single cell suspensions by passing the isolate through Nylon wool. Isolated lymphocytes were resuspended in HBSS and divided into three groups. The viability of these cells was determined by staining them with Trypan blue. A sample of 10 μM Hoechst 33342, **5**, or **6** was added to different groups, and cells were incubated for 15 min and 1 h. Treated cells were dropped on clean, chilled glass slides and mounted. The fluorescence intensity of 100 cells was measured using a Nikon fluorescence microscope and the software ImagePro Plus. The mean values of the intensity were calculated and plotted as intensity vs ligand concentration for 15 min and for 1 h.

Appendix

Abbreviations. DMA **5**, 5-(4-methylpiperazin-1-yl)-2-[2′-(3,4-dimethoxyphenyl)-5′-benzimidazolyl]benzimidazole; TBZ **6**, 5-(4-methylpiperazin-1-yl)-2-[2′{2′′-(4-hydroxy-3-methoxyphenyl)-5′′-benzimidazolyl}-5′-benzimidazolyl]benzimidazole; CTDNA, calf thymus DNA; T_m, melting temperature; Gy, gray; R_f, relative fluorescence enhancement; LET, linear energy transfer.

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