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# (54) Title: A PROCESS FOR THE SYNTHESIS OF BISBENZIMIDAZOLES AND ITS DERIVATIONS

**(57) Abstract:** A process for the synthesis of bisbenzimidazoles and its derivations comprising; (i) reacting 5 chloroaniline with zinc dust and acetic anhydride to produce 5 chloroacetanilide; (ii) reacting 5 chloroacetanilide with $HN0_3$ to produce 2-nitro-5-chloroacetanilide; (ix) adding sodium methoxide to 2-nitro-5-ch1oroaniline; (x) heating 2-nitro-5-chloroaniline, methyl piperazine, anhydrous $K_2CO_3$ and Dimethyl formamide at 100-120°C produce a mixture which is cooled by pouring ice and is filtered to obtain 5-(4'-methylpiperazin-1'-yl)-2-nitroaniline; (xi) treating 5-(4'-methylpiperazin-l'yl)-2-nitroaniline with Pd/C to produce 2-amino-4-(4'-methylpiperazin-1'-yl) aniline; (xii) refluxing a mixture of 2-amino-4-(4'-methylpiperazin-1'yl) aniline and ethyl-4-amino-3-nitrobenzenecarboximidate hydrochloride in presence of ethanol/glacial acetic acid to produce 4-[5'-(4"-methylpiperazin-1"-yl) 15 benzimidazol-2'-yl)-2-nitroaniline; (xiii) treating a solution of 4-[5'-(4"-methylpiperazin-1"-yi) benzimidazol-2'-yl)-2-nitroaniline with palladium on carbon to yield 2-amino-4-[5'-(4"-Methylpiperazin-1"-yl)benzimidazol-2'-yl]aniline; (xiv) heating 2-amino-4-[5'-(4"-Methylpiperazin-1"-yl)benzimidazol-2'-yl]aniline and 3-4-dimethoxy benzaldehyde using nitrobenzene as a solvent at 110-150°C to produce (DMA) i.e 5-(4-methylpiperazin-1-yl)-2-[2'-(3,4-dimethoxyphenyl)-5'-benzimidazolyl] benzimidazole; (ix) heating 2-amino-4-[5'-(4"-Methylpiperazin-1"-yl) benzimidazol-2'-YI] aniline and 5-Formyl-[3-methoxy-4-hydroxy benzimidazole] using nitrobenzene at 110°C to 150°C in presence of argon to produce (TBZ) i.e 5-(4-methylpiperazine-1-yl)-2-[2' (2"-(4-hydroxy-3methoxyphenyl)5"benzimidazolyl) -5'- benzimidazolyl] benzimidazole.

# A process for the synthesis of bisbenzimidazoles and its derivations.

## FIELD OF THE INVENTION

This invention relates to the process for the synthesis of bisbenzimidazoles and its derivations.

## BACKGROUND OF THE INVENTION

The DNA ligands such as bisbenzimidazoles Hoechst 33342 and Hoechst 33258, form strong and non-covalent linkages, with the adenine and thymine rich regions in the minor groove of DNA, significantly altering the chromatin structure [Exp. Cell Res. (1973), 81,474-477; Biochemistry (1990), 29,9029-9039]. The dyes Hoechst 33342 and Hoechst 33258 are frequently used in cytometry to stain chromosomes insitu. [J. Histochem. Cytochem(1985),33,333,338]. These two bis-benzimidazole compounds become brightly fluorescent when they bind to DNA. For a long time, it has been known that Hoechst 33258 binds specifically to AT-rich sequences in DNA [Biochim. Biophys. Acta 1987, 949, 158-168; J. Mol. Biol. 1987, 197, 257; Biochemistry 1991, 30, 182-189; Biochemistry 1991, 30, 10294-10306; EMBO J. 1992, 11, 225-232]. A variety of NMR and X-ray crystal structures of Hoechst 33258 bound to different oligonucleotide duplexes have been published [Biochemistry (1989) 28, 7849-7859; Nucleic Acids. Res. (1990) 18, 3753-3762; J. Chem. Soc., Chem. Commun. (1991) 1770; Eur. J. Biochem (1993) 211, 437-447; Biochemistry (1991) 30, 11377-11388]. Collectively, these structural studies reveal that the drug fits snugly into the minor groove of the double helix, covering a run of four contiguous AT base pairs. The Hoechst 33258-DNA interaction appear to be stabilized by several H-bonding and van der Waals contacts [Structure 1, 177] but in fact these molecular forces are believed to contribute to overall binding affinity [J. Mol. Biol (1997) 271, 244-257]. The hydrophobic transfer of ligand from solution on to its DNA binding sites is more likely to represent the main driving force for the complex formation [J. Mol. Biol. (1997) 271, 244-257]. Administration of these compounds prior to irradiation afford protection against the formation of primary lesions in the aqueous solutions of DNA as well as in the intact cell nucleus. These DNA ligands have also been observed to reduce the radiation – induced cytogenetic damage and cell death in cell cultures, as well as in whole body irradiated animals [Ind. J. Exp. Biol (1998) 36, 375-384; Br. J. Cancer (1989) 60, 715-721]. However, post-irradiation treatment of cells with these ligands has been observed to enhance cell death in vitro [Ind.J.Exp.Biol(1998)36,375-384]. Free radical scavenging and quenching o DNA radicals appear to be the mechanisms responsible for protection by Hoechst compounds administered prior to irradiation [Int. J. Radiation Oncol. (1992) 23, 579-586; Radioprotection (1997) 32,Cl-89], but its role in enhancing the radiation- induced cell death when administered after irradiation is not clearly understood.

The limitations of these minor groove binding ligands a being mutagenic, clatogenic and cytotoxic because of the DNA lesions caused on account of topoisomerase I inhibition, gene expression alteration and repair inhibition prevent them from being used in humans. Therefore, the development of DNA binding ligands (Minor Groove Binding Ligands particularly) that afford radioprotective effect without significant mutagenicity and cytotoxic effects can play a significant role in biological radiation protection.

## OBJECTS OF THE INVENTION

An object of the invention is to synthesize benzimidazoles and its derivatives.

Further object of this invention is to synthesize benzimidazoles having pharmacological activity.

Yet another object of this invention is to synthesize benzimidazoles DNA ligands which can afford radioprotective effect without significant mutagenicity and cytotoxic effects.

## DETAILED DESCRIPTION OF THE INVENTION

According to this invention there is provided a process for the synthesis of benzimidazoles and its derivatives comprising:

(i) reacting 5 chloroaniline with zinc dust and acetic anhydride to produce 5 chloroacetanilide;

(ii) reacting 5 chloroacetanilide with $HNO_3$ to produce 2-nitro-5-chloroacetanilide;

(iii) adding sodium methoxide to 2-nitro-5-chloroaniline;

(iv) heating 2-nitro-5-chloroaniline, methyl piperazine, anhydrouos $K_2CO_3$ and Dimethyl formamide at 100-120°C produce a mixture which is cooled by pouring ice and is filtered to obtain 5-(4'-methylpiperazin-1'-yl)-2-nitroaniline;

(v) treating 5-(4'-methylpiperazin-1'yl)-2-nitroaniline with Pd/C to produce 2-amino-4-(4'-methylpiperazin-1'-yl) aniline;

(vi) refluxing a mixture of 2-amino-4-(4'-methylpiperazin-1'yl) aniline and ethyl-4-amino-3-nitrobenzenecarboximidate hydrochloride in presence of ethanol/glacial acetic acid to produce 4-[5'-(4"-methylpiperazin-1"-yl) benzimidazol-2'-yl)-2-nitroaniline;

(vii) treating a solution of 4-[5'-(4"-methylpiperazin-1"-yl) benzimidazol-2'-yl)-2-nitroaniline with palladium on carbon to yield 2-amino-4-[5'-(4"-Methylpiperazin-1"-yl)benzimidazol-2'-yl]aniline;

(viii) heating 2-amino-4-[5'-(4"-Methylpiperazin-1"-yl)benzimidazol-2'-yl]aniline and 3-4-dimethoxy benzaldehyde using nitrobenzene as a solvent at 110-150°C to produce (DMA) i.e 5-(4-methylpiperazin-1-yl)-2-[2'-(3,4-dimethoxyphenyl)-5'-benzimidazolyl] benzimidazole;

(ix) heating 2-amino-4-[5'-(4"-Methylpiperazin-1"-yl) benzimidazol-2'-yl] aniline and 5-Formyl-[3-methoxy-4-hydroxy benzimidazole] using nitrobenzene at 110°C to 150°C in presence of argon to produce (TBZ) i.e 5-(4-methlypiperazine-1-yl)-2-[2'{2"-(4-hydroxy 3methoxyphenyl)5"benzimidazolyl} –5'- benzimidazolyl] benzimidazole.

5-Cyano-2 [3-methoxy-4-hydroxy benzimidazole] 5'-yl is produced by reacting 3,4-Diamino benzonitrile and 3-methoxy-4-hydroxy benzaldehyde. 5-Cyano-2 [3-methoxy-4-hydroxy benzimidazole] 5'-yl is then treated with Raney nickel in presence of formic acid to produce 5-Formyl-[3-methoxy-4-hydroxy benzimidazole]

The complete reaction is shown in the accompanying reaction scheme.
The pharmacological activities of two benzimidazoles having bisubstituted phenyl ring are described. The relative pharmacological activity of **5-(4-methylpiperazin-1-yl)–2-[2'-(3,4–dimethoxyphenyl)-5'-benzimidazolyl] benzimidazole (DMA)** and **5-(4-methylpiperazin-1-yl)-2-[2'{2''-(4-hydroxy3methoxyphenyl) 5''benzimidazolyl} -5'-benzimidazolyl] benzimidazole (TBZ)** was compared to that of Hoechst 33342. The two compounds on the basis of earlier observation that Hoechst 33342 is more cytotoxic than Hoechst 33258 as the para phenolic group of Hoechst 33258 is being replaced by ethoxy group in Hoechst 33342. Keeping above observations in mind, we have introduced two methoxy group in the bisbenzimidaole and one methoxy and one hydroxyl group in trisbenzimidazole. The synthesis of bisbenzimidazole was carried out basically as described by Kelly et al (1994) using 2-Amino-4-[5'-(4''-Methylpiperazin-1''-yl)benzimidazol-2'-yl]aniline and 3,4-dimethoxy benzaldehyde in 30% yield. This methodology is different from the method described by Lown et al. where they have condensed 2-arylbenzimidazole with o-arylenediamine . The terbenzimidazole was prepared using a methodology described by Edmond J. La Voie (1995) using an equimolar mixture of 2-Amino-4-[5'-(4''-Methylpiperazin-1''-yl)benzimidazol-2'-yl]aniline and 5-Formyl-[3-methoxy-4-hydroxy

benzimidazole] in nitrobenzene at 140-150$^0$C for 36 h in 25% yield. In this reaction the initially formed schiff base undergoes oxidative cyclization by nitrobenzene to give terbenzimidazole. However, we introduced two important modifications in bisbenzimidazole and terbenzimidazole. This is the first report of synthesis of terbenzimidazole having bisubstitution on the phenyl ring. In the bisbenzimidazole the two methoxy groups are introduced which are electron donating whereas in the terbenzimidazole one methoxy group was replaced by hydroxyl group to observe the stability of DNA-drug complex using hydroxyl group capable of hydrogen bonding.

Both of the above mentioned compounds are non-cytotoxic even at 100μM concentration even upto 72 hours after treatment in human glioma cell line BMG-1. Cell survival assay showed that 5-(4-methylpiperazin-1-yl)-2-[2′-(3,4-dimethoxyphenyl)-5′-benzimidazolyl] benzimidazole (DMA) has 73% cell survival at 100μM concentration whereas5-(4-methylpiperazin-1-yl)-2-[2′{2″-(4-hydroxy-3methoxyphenyl) 5″benzimidazolyl}-5′-benzimidazolyl] benzimidazole (TBZ) at lower concentration i.e., 10μM has shown increase in growth whereas at 100μM showed 92% cell survival. These results are further supported by growth kinetics.

Under the same conditions, the cells were irradiated with 2Gy, 5Gy and 10Gy of radiation and physico-chemical studies like UV-Vis spectroscopy, fluorescence spectroscopy and thermal denaturation studies were done. Cytotoxicity, cell survival assays were performed in vivo in human brain glioma cell line (BMG-1). The studies revealed that the bisubstituted minor groove binding analogues of Hoechst 33258 showed high degree of protection against high doses of radiation also.

The cytotoxic effect of DMA and TBZ on exponentially growing tumor cells BMG-1 was studied as a function of time. Cells were treated for 1 h with the ligands and allowed to grow for 24, 48 and 72 hours. The MTT assay was performed at the specified time points. It was observed that Hoechst 33342 was highly cytotoxic at 10μM concentration whereas

DMA and TBZ do not show any cytotoxicity even at 100μM concentration. There seemed to be no effect on the metabolic status of the treated cells. However, strikingly the metabolic activity of the treated cells seemed to be enhanced as compared to the untreated control cells. To further support our results we have performed cell survival assay at 0.1uM, 10uM and 100uM concentration of drug having control as untreated cell. These experiments were done thrice in six sets . Exponentially growing cells were used in these experiments and cell survival was studied using Macrocolony assay. The plating efficiency was nearly 77%. The parent compound Hoechst 33342 showed a significant effect on cell survival. At low concentrations, Hoechst 33342 do not seem to have considerable effect on cell survival whereas at 10μM concentration the surviving fraction reached remained 45% and at 100μM only 10% of cells survived. DMA at low concentration (0.1,1μM) did not show any effect on survival but at 10μM there is a slight (3%) decrease in surviving fraction and at 100μM, 73% of the cells survived. TBZ had a significantly different behavior than the two ligands. At low concentrations (upto 10μM) , TBZ seemed to have a slight stimulatory effect on growth whereas at 100μM showed a little (9%) decrease in surviving fraction. The effect of DMA and TBZ on proliferation of exponentially growing cells at 100μM concentration was also studied. The growth kinetics of the treated cells did not differ from the untreated cells. The increase in cell number as a function of time for the control as well as the treated cells remained same .

The radioprotective effect of the newly synthesized ligands were studied spectroscopically as well as in human brain glioma cell line BMG-1. The spectroscopic studies done included UV-Vis sepctroscopy, Fluorescence spectroscopy, Anisotropy measurements and Lifetime measurements and Thermal denaturation studies. Calf thymus DNA was chosen as the DNA material and ligands were incubated with the DNA and irradiated at different doses of radiation. The irradiated samples did not show any difference in the measurements as compared to the control DNA sample. The degree of DNA helix stabilization is considerably increased in the presence of the radiation and thus no DNA strand breaks were detected. At the cellular level also, the ligands were incubated with the cells and cell survival assay was performed to study the radioprotective effect of the new ligands in comparison to the parent molecule Hoechst 33342. The new ligands showed high degree of radioprotection even at lower concentrations of the ligands.

The property of being non-cytotoxic coupled with the radioprotective effect of the new ligands make them potential candidate for protection against radiation.

## Examples

**Example 1 :**

**2-Nitro-5-chloroaniline :** A solution of metallic sodium(0.012 g, 0.52mmol) in 44ml of absolute methanol was added to 2-nitro-5-chloro acetanilide (4.8 g, 23mmol). The solution was boiled under reflux for 3h and then the solvent was evaporated to obtain the desired yellow crystalline product in 98% yield. m. p. 126-128$^0$ C

**Example 2 :**

**5-(4′-methylpiperazin-1′yl)-2-nitroaniline:** A stirred mixture of 2-nitro-5-chloroaniline(3.4g, 19.76mmol), 1-methylpiperazine (4.4ml, 52mmol), anhydrous $K_2CO_3$ and dry DMF (41ml) was protected with a $CaCl_2$ drying tube and heated at 120$^0$C for 20h. Upon cooling the mixture was poured into 300ml ice cold water and filtration of the resulting suspension afforded a yellow solid. Recrystallized using $CCl_4$ to give lustrous bright yellow plates to get 4.237g (100% yield) of the title compound.

m. p. : 152$^0$ C IR : $^1$H NMR : δ 2.27(s,3H , $NCH_3$), 2.47( m,4H,H 3',5'), 6 (d, J 3Hz, 1H, H6), 6.37 (dd, J10, 3Hz, 1H, H4), 6.89(s, 2H, $NH_2$ )

**Example 3 :**

**2-Amino-4-(4′-methylpiperazin-1′-yl) aniline** : A solution of 5-(4′-methylpiperazin-1′yl)-2-nitroaniline (2.275g, 9.63mmol ) in methanol/ethyl acetate (20:80, 98 ml) was treated with 5% Pd/C ( 500 mg) and the mixture was hydrogenated at room temperature and atmospheric pressure. After the reaction is complete , the solution is colorless. Filtration (celite) and concentration of the filtrate without delay afforded the pale yellow colored diamine (4a) in 100% yield.

**Example 4 :**

**4-Amino-3-nitrobenzonitrile:** 4-cyano-2-nitroacetanilide (3g,15mmol) was heated under reflux in 10% $H_2SO_4$ for 30 min. Upon cooling the resulting precipitate was filtered off and dried. Recrystallization of this material (methanol/water) gave yellow crystals of 4-amino-3-nitro benzonitrile in 95% yield. m. p. 160$^0$C

**Example 5:**

**Ethyl-4-Amino-3-nitrobenzenecarboximidate hydrochloride** : 4-amino-3-nitrobenzonitrile (2g ,12.5mmol) was suspended in dry ethanol (200ml at 10mg/ml concentration)and cooled in an ice/water bath. Anhydrous HCl gas was bubbled rapidly through the mixture for 30-45 min. During this time, dissolution followed by rapid precipitation was observed. The vessel was fitted with a $CaCl_2$ drying tube and the thick suspension was stirred overnight. The ethanol was removed by rotary evaporation and the pasty residue was triturated with dry diethyl ether. Filtration, followed by drying under reduced pressure afforded the title imino ether hydrochloride as a hygroscopic bright yellow powder in 80 % yield. m. p. 233$^0$C; IR : 3469, 3382,3340, 2227, 1631, 1556, 1271, 920, 821, 765 cm $^{-1}$;$^1$H NMR : δ 1.46( t, J 7Hz,3H, $OCH_2CH_3$ ), 4.58 (q, J7Hz, 3H, $OCH_2CH_3$ ), 7.16( d, J 9Hz, 2H, H6), 8.79( d, J 2Hz , 1H, H2)

**Example 6 :**

**4-[5′-(4″-methylpiperazin-1″-yl)benzimidazol-2′-yl]-2-nitroaniline** : A mixture of freshly prepared 2-Amino-4-(4′-methylpiperazin-1′-yl) aniline (2.01 g, 9.75mmol) and Ethyl-4-Amino-3-nitrobenzenecarboximidate hydrochloride (1.98 g, 9.5mmol) in dry ethanol/ glacial acetic acid (2:1,69 ml) was maintained under nitrogen and heated at reflux for 4 h. The mixture was cooled and then concentrated to afford an orange pasty residue. This material was dissolved in water and addition of conc. Ammonia solution resulted in precipitation of a solid; the suspension was allowed to stand overnight. The brick red solid was collected by filtration and washed thoroughly with water before being dissolved in acetic acid/ methanol (7.5: 92.5), 50 ml. The deep red solution was filtered and then made alkaline with conc. Ammonia solution (c. 20ml) .A fine orange precipitate formed immediately and the suspension was allowed to stand for several hours before filtration. The solid thus retained was washed thoroughly with water and then with acetone. Drying under reduced pressure afforded an orange solid in 74% yield. m. p. 184-186$^0$ C;

IR : 3494, 3365, 2939, 2808, 1639, 1510, 1247, 796 cm $^{-1}$;$^{1}$H NMR : δ 2.99(s,3H, NCH$_3$), 3.16 (m,2H), 3.34 (m,2H), 3.67 (m,2H), 3.94 (m,2H), 7.24 (d,J9Hz,1H), 7.25(d, J2Hz, 1H;), 7.36 (dd, J9Hz, 1H), 7.98 ( dd, J 9.2 Hz, 1H), 8.95 (d, J 2Hz, 1H)

**Example 7 :**

**2-Amino-4-[5′-(4″-Methylpiperazin-1″-yl)benzimidazol-2′-yl]aniline :**

A solution of 4-[5′-(4″-methylpiperazin-1″-yl)benzimidazol-2′-yl]-2-nitroaniline (1.04 g ,30mmol) in ethyl acetate/methanol (80 ml of 2:1 mixture) was treated with 5% palladium on carbon (250 mg) and hydrogenated at room temperature and atmospheric pressure. When hydrogen uptake has ceased , the solution was filtered (celite) and concentrated without delay to afford the orange-brown colored diamine .

**Example 8 :**

**5-Cyano-2 [3-methoxy-4-hydroxy benzimidazole] 5′-yl :** A solution of 1.224 g (9.2 mmol) of previously obtained 3,4-Diamino benzonitrile , 1.4 g (9.2mmol) of 3-methoxy-4-hydroxy benzaldehyde in nitrobenzene are taken in a three necked round bottom flask under nitrogen and heated at 140$^0$ C. The reaction mixture is heated for 18 hours with stirring, nitrobenzene is then removed under reduced pressure to obtain the brown colored crude product (14). The final product is then obtained through silica gel (60-120 mesh size) column chromatography using EtOAc / MeOH as eluent. m.p. 226-230$^0$ C; IR 3425.3 (O-H), 3263.3 (-NH), 2221.8 (-CN), 1278.7 (C-O-C) cm$^{-1}$; $^1$H NMR δ 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$_3$)

**Example 9 :**

**5-Formyl-[3-methoxy-4-hydroxy benzimidazole] :** To a solution of 1 g (3.77mmol) 5-Cyano-2 [3-methoxy-4-hydroxy benzimidazole] 5′-yl in 60ml formic acid and 20 ml of water, Raney nickel (3.96 g) was added . The reaction mixture was heated at 95$^0$ C for 6 hours. The hot mixture was filtered (celite) and the reaction flask and the celite bed were rinsed with water. The aqueous solution was concentrated to dryness. To this residue, water is added 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 ($Na_2SO_4$) and concentrated in vacuo to give the yellow colored compound in 30% yield. m. p. 258 –$260^0$C ; IR 3435.6 (-OH) ,3194.55 (NH) , 1675.6 (CHO), 1594, 1506, 1441.6, 1280.7cm $^{-1}$ ; $^1$H NMR δ 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, -$OCH_3$)

**Example 10 :**

**5-(4-methylpiperazin-1-yl)-2-[2'-(3,4-dimethoxyphenyl)-5'-benzimidazolyl] benzimidazole** : A solution of freshly prepared 2-Amino-4-[5'-(4''-Methylpiperazin-1''-yl)benzimidazol-2'-yl]aniline (1.18g,3.67mmol) and 3,4-dimethoxy benzaldehyde ( 0.61g ,3.67mmol) in nitrobenzene (110 ml) is heated at 140-$150^0$ C for 24 h. The solvent is then removed under reduced pressure to give the final crude product as a brown colored solid. The product is purified by column chromatography on BUCHI 688 Liquid (MPLC) Pump using silica gel (70-230 mesh size) and EtOAc/ MeOH as eluent to give a yellow colored compound and characterized by spectroscopic techniques .m. p. $220^0$C Yield : 30%; IR : 3556, 2922, 1629, 1508, 1417, 1371, 1296, 1022, 810cm$^{-1}$; $^1$H NMR (DMSO-$d_6$) δ 2.24 (s,3H,$NCH_3$), 2.46 (t,4H,$CH_2$,J=4Hz), 3.5 (t,4H,$CH_2$, J=4Hz), 3.86 (s,3H,$OCH_3$), 6.77 (d,1H,Ar-h,J=8Hz), 7.13 (d,2H,Ar-H, J= 9Hz), 7.64 (d,1H,Ar-H,J=8.5Hz ), 7.78 (d,1H,Ar-H,J=8 Hz), 8.03 (m,1H,Ar-H),8.17(d,2H,Ar-h, J=9Hz), 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

**Example 11 :**

**5-(4-methylpiperazin-1-yl)-2-[2'{2''-(4-hydroxy-3methoxyphenyl)5''benzimidazolyl} -5'-benzimidazolyl] benzimidazole** : A solution of freshly prepared 2-Amino-4-[5'-(4''-Methylpiperazin-1''-yl)benzimidazol-2'-yl]aniline (218 mg, 0.82mmol) and 5-Formyl-[3-methoxy-4-hydroxy benzimidazole] (182 mg ,0.68mmol) in nitrobenzene is taken under argon in a round bottom flask and heated at 140$^0$C for 20 hours. Nitrobenzene is removed under reduced pressure and the resulting solid is purified by column chromatography (EtOAc/ MeOH) on BUCHI 688 Liquid (MPLC) Pump using silica gel (70-230 mesh size) to obtain the final product (25% yield) as a brown colored solid. m.p. >290$^0$C ; IR 3435, 3195, 1632, 1560, 1413, 1281; $^1$H NMR δ 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 of TBZ was observed on MALDI : 570.3

**Cytotoxicity Assay**

The cytotoxicity was determined using the MTT microtiter plate tetrazolium cytotoxicity assay (MTA). The human brain malignant glioma cell line BMG-1 was used. The cytotoxicity assay was performed using 96-well microtiter plates. 3000 cells/ well were plated and treatment of the ligands was done 24 hours post plating. For determination of $IC_{50}$ , cells were exposed continuously with varying concentration of drug and MTT assays were performed at the end of fourth day. To the control and treated cells , 20 µl of 5mg/ml MTT in PBS was added, incubated for 2 hours at 37$^0$C and then the medium was removed. 150 µl of DMSO was added and the plate was read at 540 nm with reference at 630 nm .

**Macrocolony Assay**

Cells were washed with HBSS and harvested using 0.05% trypsin. Depending on the treatments, 200-1200 cells were plated in 90-mm Petri dishes and incubated at 37$^0$C in a 5% $CO_2$ humidified atmosphere for 8-10 days. Colonies were fixed in methanol and stained with 1% crystal violet. Colonies containing more than 50 cells were counted as shown in Table 1&2.

**Proliferation Kinetics** BMG-1 cells were seeded at 7000-8000 cells/ cm$^2$, and their proliferation kinetics was measured at 24-h intervals by trypsinizinig and counting total cells per flask using a hemocytometer.

**Table 1**

## Thermal Denaturation studies of ligand-DNA complex in presence and absence of radiation

| Ligand | Ratio [ligand]/[DNA] | Dose (Gy) | Tm ($^0$C) |
|---|---|---|---|
| TBZ | 0.01 | 0 | 74.3 |
| | | 100 | 75 |
| | 0.1 | 0 | 77.7 |
| | | 100 | 81.5 |
| Hoechst 33258 | 0.01 | 0 | 71.5 |
| | | 100 | 67.5 |
| | 0.1 | 0 | 81.7 |
| | | 100 | 79.3 |

## Table 2

### Cell Survival Assay in BMG-1 cells with and without irradiation

| Treatment | Plating efficiency % | Survival fraction |
|---|---|---|
| Control | 67 | 1 |
| DMA (1uM) | 65 | 0.97 |
| DMA(10uM) | 67 | 1 |
| DMA (100uM) | 64 | 0.95 |
| TBZ (1uM) | 73 | 1.07 |
| TBZ (10uM) | 71 | 1.05 |
| TBZ (100uM) | 69 | 1.02 |
| 2Gy | 51 | 0.75 |
| 5Gy | 15 | 0.22 |
| 10Gy | 2.0 | 0.03 |
| DMA (10uM) + 2 Gy | 63 | 0.93 |
| TBZ (10uM) + 2Gy | 60 | 0.89 |
| DMA (1uM) +5 Gy | 22 | 0.32 |
| DMA (10uM) + 5 Gy | 27 | 0.40 |
| DMA (100uM) + 5 Gy | 25 | 0.38 |
| TBZ (1uM) + 5Gy | 21 | 0.31 |
| TBZ (10uM) + 5Gy | 20 | 0.30 |
| TBZ (100uM) + 5 Gy | 18 | 0.26 |
| DMA (10uM) + 10 Gy | 4 | 0.06 |
| TBZ (10uM) + 10 Gy | 3.5 | 0.05 |

**Experiment with Normal human embryonic kidney cell line (Hek cell line):**

To demonstrate the effect of DMA and TBZ as radioprotectors in comparison to Hoechst 33342, we also chose a normal (untransformed) human embryonic kidney cell line. Though the two cell lines, Hek and BMG-1 cell line are not radiobiologically different, it was still reasonable to demonstrate the *invitro* effect of these ligands on different cell lines.

The results with the two cell lines are not significantly different, but it can be concluded that both DMA and TBZ are better radioprotectors in comparison to Hoechst 33342.

Below presented are the results of the growth kinetics and Macrocolony assay performed with the two cell lines.

**Procedure:**

**Macrocolony Assay (Cell Survival Assay)**

Both cell lines in triplicate were washed with HBSS, plated and grown for four days before harvesting using 0.05% trypsin. Depending on the treatments, 200 to1200 cells were plated in 90 mm petri dishes and incubated at $37^0$C in 5% $CO_2$ humidified atmosphere for 8 to10 days. Colonies were fixed with methanol and stained with 1 % crystal violet. Colonies containing more than 50 cells were counted.

**Proliferation Kinetics**

Both the cell lines in triplicates were seeded at 7000 to 8000 cells/ $cm^2$, and their proliferation kinetics was studied at 24 h intervals following trypsinization and counting total cells per flask using a haemocytometer.

**Irradiation Procedures:**

Exponentially growing cells, 24 h after plating were irradiated at room temperature in growth medium with a Co-60 source (Gamma cell, AECL, Canada) at a dose rate of 0.5 to1.2 Gy/min. Concentration of 10µM drug was added 1h before irradiation. Following this procedure, cells were grown for various time intervals to study growth kinetics and plated for Macrocolony assay as shown in the following Tables 3& 4 and figure 1 & 2

## Table 3

### Cell Survival Assay in BMG-1 cells with 5 Gy irradiation

| Treatment | Plating efficiency % | Survival fraction |
|---|---|---|
| Control | 67 | 1 |
| 5Gy | 15 | 0.22 |
| DMA (10uM) + 5 Gy | 27 | 0.40 |
| TBZ (10uM) + 5 Gy | 20 | 0.30 |
| Hoechst 33342 + 5Gy | 20 | 0.10 |

## Table 4

### Cell Survival Assay in Hek cells with 5Gy irradiation

| Treatment | Plating efficiency % | Survival fraction |
|---|---|---|
| Control | 70 | 1 |
| 5Gy | 15 | 0.22 |
| DMA (10uM) + 5 Gy | 27 | 0.50 |
| TBZ (10uM) + 5 Gy | 25 | 0.48 |
| Hoechst 33342 + 5Gy | 20 | 0.10 |

WE CLAIM

1. A process for the synthesis of bisbenzimidazoles and its derivations comprising:

(i) reacting 5 chloroaniline with zinc dust and acetic anhydride to produce 5 chloroacetanilide;

*(ii) reacting 5 chloroacetanilide with* $HNO_3$ *to produce* 2-nitro-5-chloroacetanilide;

(iii) adding sodium methoxide to 2-nitro-5-chloroaniline;

(iv) heating 2-nitro-5-chloroaniline, methyl piperazine, anhydrous $K_2CO_3$ and Dimethyl formamide at 100-120°C produce a mixture which is cooled by pouring ice and is filtered to obtain 5-(4'-methylpiperazin-1'-yl)-2-nitroaniline;

(v) treating 5-(4'-methylpiperazin-1'yl)-2-nitroaniline with Pd/C to produce 2-amino-4-(4'-methylpiperazin-1'-yl) aniline;

(vi) refluxing a mixture of 2-amino-4-(4'-methylpiperazin-1'yl) aniline and ethyl-4-amino-3-nitrobenzenecarboximidate hydrochloride in presence of ethanol/glacial acetic acid to produce 4-[5'-(4"-methylpiperazin-1"-yl) benzimidazol-2'-yl)-2-nitroaniline;

(vii) treating a solution of 4-[5'-(4"-methylpiperazin-1"-yl) benzimidazol-2'-yl)-2-nitroaniline with palladium on carbon to yield 2-amino-4-[5'-(4"-Methylpiperazin-1"-yl)benzimidazol-2'-yl]aniline;

(viii) heating 2-amino-4-[5'-(4"-Methylpiperazin-1"-yl)benzimidazol-2'-yl]aniline and 3-4-dimethoxy benzaldehyde using nitrobenzene as a solvent at 110-150°C to produce (DMA) i.e 5-(4-methylpiperazin-1-yl)-2-[2'-(3,4-dimethoxyphenyl)-5'-benzimidazolyl] benzimidazole;

(ix) heating 2-amino-4-[5'-(4"-Methylpiperazin-1"-yl) benzimidazol-2'-yl] aniline and 5-Formyl-[3-methoxy-4-hydroxy benzimidazole] using nitrobenzene at 110°C to 150°C in presence of argon to produce (TBZ) i.e 5-(4-methlypiperazine-1-yl)-2-[2'{2"-(4-hydroxy-3methoxyphenyl)5"benzimidazolyl} –5'- benzimidazolyl] benzimidazole.

2. A process as claimed in claim 1 wherein the said 5-Formyl-[3-methoxy-4-hydroxy benzimidazole] is prepared by reacting 5-Cyano-2[3-methoxy-4-hydroxy benzimidazole] 5-yl with Raney Nickel in presence of formic acid at 95°C for 6 hours.
3. A process as claimed in claim 1, wherein said 5-Cyano-2[3-methoxy-4-hydroxy benzimidazole] 5-yl is prepared by reacting 3,4-Diaminobenzonitrile and 3-methoxy-4-hydroxy benzaldehyde in nitrobenzene at 140°C.
4. A process as claimed in claim 1, wherein 2-Nitro-5-chloroaniline is prepared by boiling sodium methoxide and 2-Nitro-5-chloro acetanilide under reflux for 3 hours.
5. A process as claimed in claim 1, wherein 5-(4'-methylpiperazin-1'yl)-2-nitroaniline is prepared by heating a mixture of 2-Nitro-5-chloroaniline, 1-methylpiperazine anhydrous $K_2CO_3$ and dry Dimethyl formamide (DMF) at 120°C for 20 hours and then cooling the mixture with ice cold water.
6. A process as claimed in claim 1, wherein 2-Amino-4-(4'-methylpiperazin-1'yl) aniline is prepared by treating 5-(4'-methylpiperazin-1'yl)-2- nitroaniline with 5%. Pd/C in the presence of methanol /ethylacetate and is hydrogenated at room temperature and at atmospheric pressure.
7. A process as claimed in claim 1, wherein Ethyl-4-amino-3-nitrobenzenecarboximidate is preparing by suspending 4-amino-5-nitrobenznitrile in dry ethanol, cooling the suspension in an ice/water bath and HCl gas was bubbled rapidly through the mixture for 30-45 minutes, the ethanol was removed by evaporation and the residue was triturated with diethyl ether followed by filtration.
8. A process as claimed in claim 7, wherein the said 4-amino-3-nitrobenzonitrile was provided by heating under reflux 4-cyano-2-nitro acetanilide in $H_2SO_4$ for 30 minutes.

9. A process as claimed in claim 1, wherein the said 4-[5'-(4"-methylpiperazin-1"-yl) benzimidazol-2'-yl]-2 nitroaniline is prepared by mixing freshly prepared 2-amino-4-(4'methylpiperazin-1'yl)aniline and Ethyl-4-amino-3-nitrobenzenecarboximidate hydrochloride in dry ethanol/glacial acetic acid under nitrogen and heating said mixture under reflux for 4 hours, the mixture is then cooled and concentrated.
10. A process as claimed in claim 1, wherein said 2-amino-4-[5'-(4"-Methylpiperazin-1"-yl)benzimidazol-2'yl] aniline is prepared by treating 4-[5'-(4"-methylpiperazin-1"-yl) benzimidazol-2'-yl]-2-nitroaniline in ethylacetate/methanol with 5% palladium on carbon and hydrogenated at room temperature and atmospheric pressure.
11. A process as claimed in claim 1, wherein said 5-(4- methylpiperazin-1-yl)-2-[2'-(3,4-dimethoxyphenyl)-5'(benzimidazolyl] benzimidazole is prepared by heating freshly prepared 2-Amino-4-[5'-(4"-Methylpiperazin-1"-yl)benzimidazol-2'-yl]aniline and 3,4-dimethoxy benzaldehyde in nitrobenzene at 140°-150°C for 24 hours and then removing the solvent under reduced pressure to give the product.
12. A process as claimed in claim 1, wherein said 5-(4- methylpiperazin-1-yl)-2-[2"-(4-hydroxy-3-methoxyphenyl)-5" benzimidazolyl] –5'-benzimidazolyl] benzimidazole is prepared by heating a freshly prepared 2-Amino-4-[5'-(4"-Methylpiperazin-1"-yl)benzimidazol-2'-yl]aniline and 5-Formyl-[3-methoxy-4-hydroxy benzimidazole] in nitrobenzene under argon in a flask at 140°C for 20 hours then nitrobenzene is removed under pressure to give the solid.
13. 5-(4- methylpiperazin-1-yl)-2-[2'-(3,4-dimethoxyphenyl)-5-benzimidazolyl] benzimidazole, DMA as produced by the process as claimed in claim 1.
14. 5-(4- methylpiperazin-1-yl)-2-[2'-2"-(4-hydroxy-3-methoxyphenyl)-5" benzimidazolyl] –5'-benzimidazolyl] benzimidazole, TBZ as produced by the process as claimed in claim 1.
15. 2-Nitro-5-chloroaniline as produced by the process as claimed in claim 1.

16. 5-(4'- methylpiperazin-1-yl)-2-nitroaniline as produced by the process as claimed in claim 1.
17. 2-Amino-4-(4'-methylpiperazin-1'-yl) aniline as produced by the process as claimed in claim 1.
18. 4-Amino-3-nitrobenzonitrile as produced by the process as claimed in claim 8.
19. Ethyl-4-Amino-3-nitrobenzenecarboximidate hydrochloride as produced by the process as claimed in claim 7.
20. 4-[5'-(4''-methylpiperazin-1''-yl) benzimidazol-2'-yl]-2-nitroaniline as produced by the process as claimed in claim 1.
21. 2-Amino-4-[5'-(4''-Methylpiperazin-1''-yl)benzimidazol-2'-yl]aniline as produced by the process as claimed in claim 1.
22. 5-Cyano-2[3-methoxy-4-hydroxy benzimidazole]5'-yl as produced by the process as claimed in claim 3.
23. 5-Formyl-[3-methoxy-4-hydroxy benzimidazole] as produced by the process as claimed in claim 2.

# Results of Proliferation kinetics of BMG-1 and Hek cell line against 5Gy radiation dose

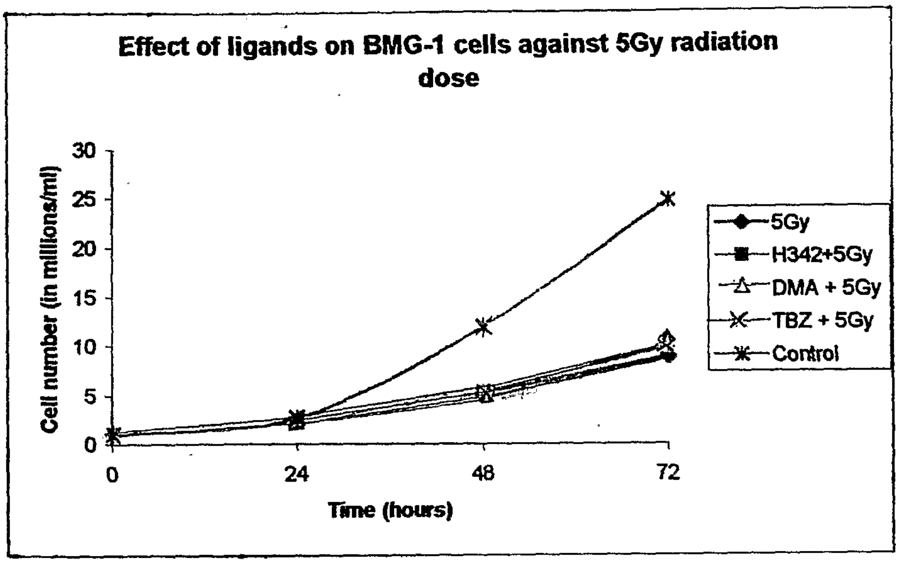

Figure 1

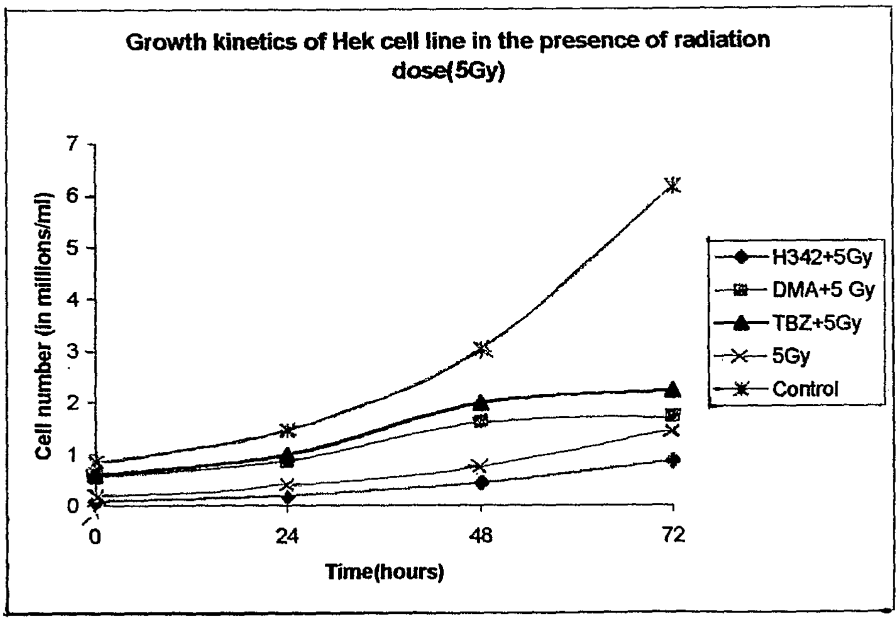

Figure 2

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN 03/00301-0

CLASSIFICATION OF SUBJECT MATTER

IPC$^7$: C07D 235/18, 235/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC$^7$: C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPOQUE; STN Karlsruhe/Registry, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | Chemical Communications, Cambridge, 1997, no. 4, pages385-386, SADAT S. E.:"Unique binding site for bis-benzimidazoles on transfer RNA" & abstract CA [online], Chemical Abstracts online [retrieved on 10 December 2003 (10.12.03) ]. Retrieved from: CA Database, STN-International, Karlsruhe (DE), CA Accession no. 126:258426 | 1-23 |
| A | Bioorganic & Medicinal Chemistry 2001, vol. 9, no. 11, pages 2905-2919; JI Yu-Hua "Tris-benzimidazole derivatives: design, synthesis and DNA sequence recognition" & (abstract) CA [online], Chemical Abstracts online [retrieved on 10 December 2003 (10.12.03) ]. Retrieved from: CA Database, STN-International, Karlsruhe (DE), CA Accession no. 136:131135 | 1-23 |

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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| | |
|---|---|
| Date of the actual completion of the international search<br>12 December 2003 (12.12.2003) | Date of mailing of the international search report<br>11 February 2004 (11.02.2004) |
| Name and mailing adress of the ISA/AT<br>Austrian Patent Office<br>Dresdner Straße 87, A-1200 Vienna<br>Facsimile No. 1/53424/535 | Authorized officer<br>KOLLER G.<br>Telephone No. 1/53424/458 |

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | Journal of Medicinal Chemistry 2003, vol. 46 no. 18, pages 3785-3792; TAWAR U. "Influence of Phenyl Ring Disubstitution on Bisbenzimidazole and Terbenzimidazole Cytotoxicity: Synthesis and Biological Evaluation as Radioprotectors"; & (abstract) CA [online], Chemical Abstracts online [retrieved on 10 December 2003 (10.12.03) ]. Retrieved from: CA Database, STN-International, Karlsruhe (DE), CA Accession no. 139:261219<br>---- | 1-23 |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal application No.
PCT/IN 03/00301-0

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| A | | none | |