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ScienceDirect
Bioorganic & Medicinal Chemistry 14 (2006) 6444–6452
Bioorganic & Medicinal Chemistry

Triple helix stabilization by covalently linked DNA–bisbenzimidazole conjugate synthesized by maleimide–thiol coupling chemistry

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Received 7 March 2006; revised 17 May 2006; accepted 18 May 2006
Available online 12 June 2006

Abstract—Tethering of $BBZPNH_2$, an analogue of the Hoechst 33258, with a 14 nucleotide long DNA sequence with the help of succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), a heterobifunctional crosslinking reagent, using DMF/water as solvent yields a conjugate which effectively stabilizes the triple helix. The above conjugate was hybridized with 26 bp long double stranded (ds) DNA having 14 bp long polypurine–polypyrimidine stretch to form a pyrimidine motif triple helix. The above conjugate increases the thermal stability of both the transitions, that is, triple helix to double helix by 12 °C and double helix to single strand transition by 16 °C for the triple helix formed with conjugated TFO over the triple helix made from non-conjugated TFO. Fluorescence and circular dichroism spectra recorded at different temperatures confirm the presence of minor groove binding bisbenzimidazole in the AT-rich minor groove of dsDNA even after the major groove bound TFO separates out.

1. Introduction

Triplex forming oligonucleotides (TFOs) have been widely used for the sequence specific targeting of dsDNA to regulate the gene expression of culprit gene to control genetic diseases by antisense/antigene approach.[1–4] In the antigene approach the TFO binds sequence selectively in the major groove of the target duplex to form the pyrimidine motif or purine motif triple helices,[5,6] but the triple helices have the limited stability under the physiological conditions.[7] To circumvent this, several strategies have been proposed to enhance the stability of the triple helical complexes, for example, the use of polycations like spermine,[8] bivalent cations[9] and the use of chemically modified nucleotides.[10,11]

Several DNA binding ligands have also been studied for the triple helix stabilization. Benzopyridoindoles and benzopyridoquinoxalines were the first molecules reported to bind to triple helix more tightly than the double helix.[12] Another promising triplex stabilizing ligand is coralyne.[13] DNA minor groove binding ligands also interact with DNA triple helix, and some of them can be used to induce the formation of triplex structures that could not be formed otherwise.[14] Berenil was found to stabilize the DNA and RNA triplexes under certain conditions.[15] SN-18071 was also found to stabilize the triple helix while the analogous compound SN-6999 was found to destabilize the triplexes.[16] Generally minor groove binding ligands tend to destabilize the DNA and RNA triplexes in the non-conjugated state.[17,18] Intermolecular triplexes could be additionally stabilized if the third strand is conjugated with a DNA binding ligand, particularly at the 5′-end of the TFO. Several intercalators,[19,20] polycations such as polyamides and polyamines,[21,22] cholesterol,[23] etc., have been tethered with the DNA to stabilize the triplexes.

Hoechst 33258 has been found to destabilize the triple helix containing the T.A.T triplets at least under the conditions examined,[18] but it stabilizes the triple helix when conjugated with the TFO at the 5′-end.[24] Recently reported DNG-Hoechst 33258 conjugate was synthesized and used to stabilize the triple helix.[25] These conjugates have been synthesized by phosphoramidite

Keywords: Triple helix; Heterobifunctional; Crosslinking reagent; Maleimide conjugate; Induced CD signal; Minor groove binders.
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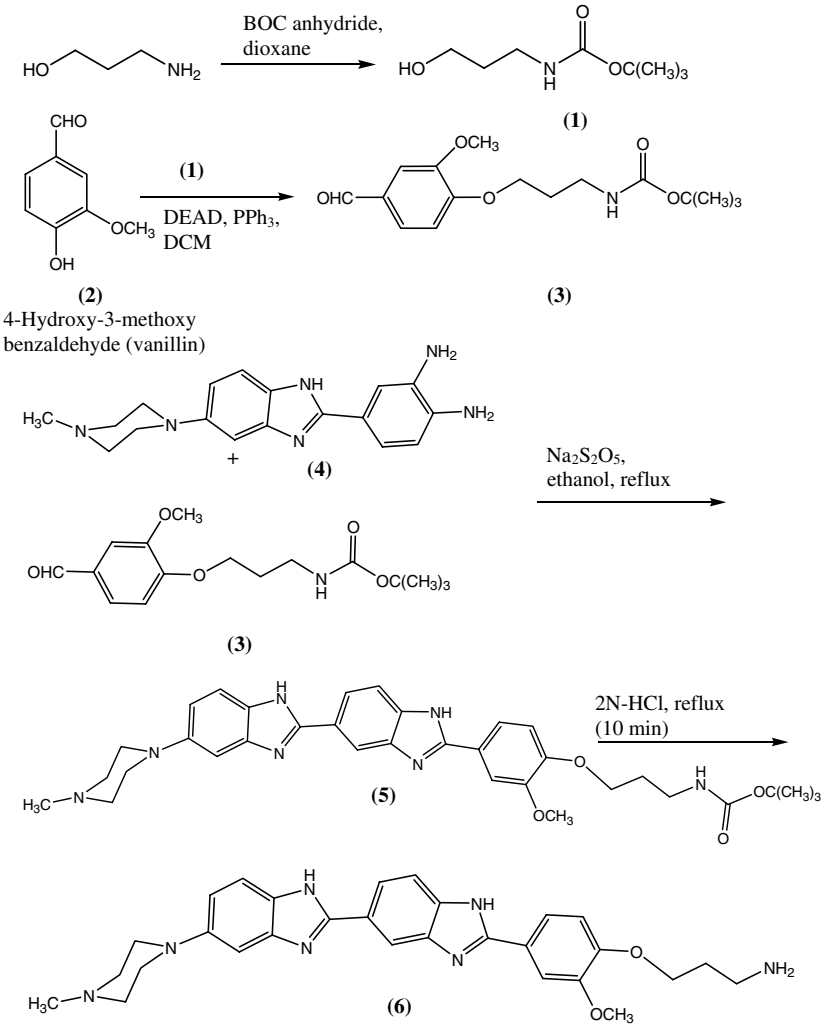
doi:10.1016/j.bmc.2006.05.034

chemistry using 'postsynthetic protocol.'[26,27] Phosphoramidites are moisture sensitive and hence their preparation requires extremely dry conditions. The heterobifunctional crosslinking reagents serve as alternate for phosphoramidite chemistry for conjugating two molecules having different chemical groups. The term heterobifunctional derives from the fact that the linkers possess functional groups capable of reacting with two distinct functional groups, for example, amines and thiols.[28] The linkers serve two purposes, first one is to covalently bind two distinct chemical entities which otherwise would remain unreactive to each other and the other one is to act as a physical spacer which provides greater accessibility and/or freedom to each of the linked molecules. Succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) provides an easy method to conjugate two molecules one having a primary amino group and other having a free –SH group in a one-pot Michael addition reaction. It has been widely used to prepare ricin-monoclonal antibody,[29] DNA–enzyme,[30] peptide–DNA,[31] and peptide–PNA[32] conjugates, but it was not used to prepare DNA–small molecule conjugates yet.

In the current study, we have synthesized an analogue of Hoechst 33258 which has bisubstitution on the terminal phenyl ring and a free primary amino group at the side chain and conjugated this bisbenzimidazole with the free –SH group of a 14mer DNA sequence with the help of SMCC using solution-phase chemistry in DMF–water by a unique methodology. This conjugate would have better cell membrane permeability than the naked DNA owing to the observation that Hoechst dyes can cross the cell membrane and nuclear membrane.[33] These types of conjugates can also be promising for developing transcription factor inhibitor[34] owing to simultaneous major and minor groove binding.

2. Results and discussion

TFO binds sequence specifically in the major groove of target dsDNA by forming Hoogsteen or reverse



5-(4-Methylpiperazin-1-yl)-2-[2'-(4-{3-aminopropyloxy}- 3-methoxyphenyl)-5'-benzimidazol]benzimidazole

Scheme 1.

Hoogsteen hydrogen bonds and form a local triple helix. Since the TFO occupies the major groove, the minor groove remains largely unoccupied. DNA minor groove binding ligand Hoechst 33258 has been tethered to the 5′-end of TFO to enhance the triple helix stability by simultaneous minor groove binding.[24] Generally 'post-synthetic' protocols have been used to prepare such conjugates.[35,36] McLaughlin and coworkers attempted to prepare a phosphoramidite of the hexaethyleneglycol derivative of Hoechst 33258.[36] They found that the phosphoramidite could not be prepared under a variety of conditions and reasoned that the benzimidazole rings were participating in a competing nucleophilic reaction with the phosphochloridite used to prepare the phosphoramidite. So they have used the 'reverse coupling' protocol to give the final conjugates in 50–75% yield in two successive coupling reactions.[37] $BBZPNH_2$–maleimide–DNA conjugate prepared in our laboratory has synthetic and biological advantages over oligonucleotide-minor groove binding drug conjugates reported till date. It is very well known that the phosphoramidite and phosphochloridite reagents are highly moisture sensitive. Hence this method has two major disadvantages, first the tedious synthesis of phosphoramidite and second the conjugation between DNA and small molecule. The approach adopted by us is relatively much simpler. $BBZPNH_2$ having a primary amino group on the side chain was reacted with SMCC to form amide bond from succinimidyl side. This amide having the reactive maleimide group was then reacted with the DNA having free

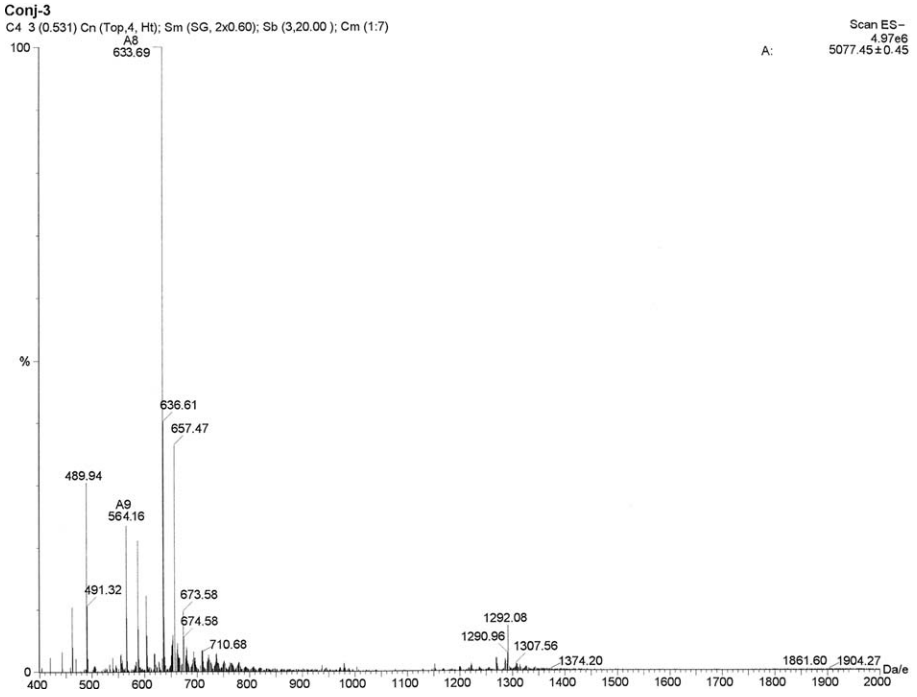


Figure 1. ESMS of bisbenzimidazole–maleimide–DNA conjugate (B).

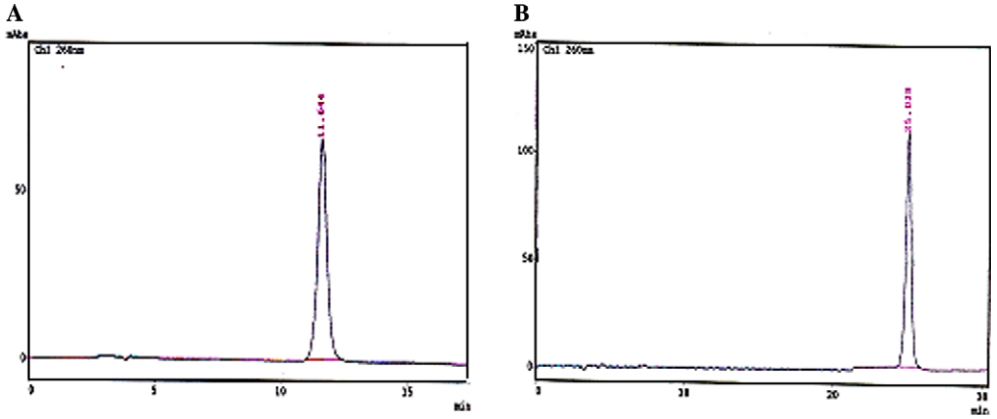


Figure 2. HPLC analysis of 14mer DNA sequence having free thiol group at the 5′-end used to make the conjugate (A) and the $BBZPNH_2$–malemide–DNA conjugate (B).

–SH group at the 5′-end to give the title conjugate in a single step.

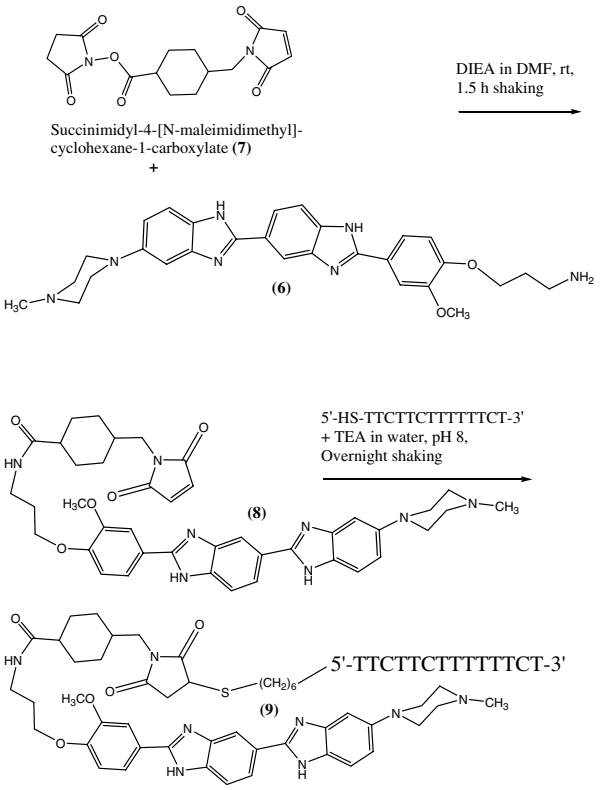
2.1. Synthesis of 5-(4-methylpiperazin-1-yl)-2-[2′-(4-{3-aminopropyloxy}-3-methoxyphenyl)-5′-benzimidazolyl]-benzimidazole (6)

3-*N*-(*tert*-Butyloxycarbonyl)-propan-1-ol **1** was reacted with vanillin **2** using Mitsunobu protocol[38] to generate the aldehyde which was then reacted with the 2-amino-4-[5′-(4″-methylpiperazin-1″-yl)benzimidazol-2′-yl]aniline (**4**),[39] to give the bisbenzimidazole **5** in which the amino group of the linker was protected with the *tert*-butyloxycarbonyl group which was removed by refluxing the solution in 2 N HCl[40] to yield **6** (Scheme 1).

2.2. Synthesis of bisbenzimidazole–maleimide–DNA conjugate

Compound **6** was reacted with SMCC, a heterobifunctional crosslinking reagent. It reacts with the compounds having free primary amino groups from the succinimide side by forming the amide bond and its maleimide moiety reacts with the free –SH group of the thiol compounds.[28] SMCC was reacted with **6** in DMF/water solution to give the bisbenzimidazole–maleimide conjugate **8**. Its molecular weight was found to be 730 by ESMS and it was reacted with free thiol group of the 14mer DNA sequence to give the final conjugate **9**. The 14mer DNA sequence having free thiol group at the 5′-end used to make this conjugate had shown a retention time of 11.64 min (Fig. 2A), while the final conjugate has a retention time of 25.02 min (Fig. 2B) under the same HPLC conditions. The molecular weight of the final conjugate was 5077.5 as confirmed by ESMS (Fig. 1, $[M]^{8-}$ peak) and the purity was confirmed by HPLC analysis (Scheme 2).

The synthesis of this conjugate was done in aqueous conditions at room temperature unlike the phosphoramidite chemistry. Conjugate **9** was obtained in 70% yield, as analyzed by HPLC in single pot synthesis using SMCC, unlike the 50–70% yield in two repetitive reactions in the case of phosphoramidite method.[37] This conjugate may serve as a better antigene therapeutical agent because of the following properties: It has a 21 atom long linker between bisbenzimidazole and DNA sequence. In a triple helix the third strand would be located in the major groove and the tethered bisbenzimidazole would go and bind in the minor groove.[24] This linker is long enough to satisfy the conditions. The thioether linkage used in conjugation is thermally stable.[41] This conjugate is supposed to have better cell membrane permeability than the naked DNA since it contains bisbenzimidazole moiety, which is known for their cell membrane and nuclear membrane permeability,[32,42] and the linker used in the synthesis contains a peptide bond, maleimide moiety, and a thioether linkage to enhance the cell permeability of the conjugate. The bisbenzimidazole dye used in the conjugate has the bisubstitution on the terminal phenyl ring. In our previous work we have shown that bisubstitution on phenyl ring makes it less cytotoxic than the parent compounds,[43] hence this conjugate may serve as better therapeutical agents.



Scheme 2.

2.3. Thermal melting studies of bisbenzimidazole–maleimide–DNA–$(DNA)_2$ triple helix complex

The duplex used in the study contains the AT-rich stretch at the target site. The triple helix formed between nonconjugated 14mer TTCTTCTTTTTTCT and 26mer DNA duplex has two transition temperatures, the T_m value for triplex to duplex transition was 32 °C and for duplex to coil transition 53 °C. There is an increase in T_m values of both transitions for the triple helix formed between bisbenzimidazole–maleimide–DNA conjugate and duplex DNA. Triplex to duplex transition was stabilized by 12 °C and duplex to coil transition was stabilized by 16 °C (Fig. 3). From these results, it is clear that conjugate is stabilizing the triple helix by simultaneous major and minor groove binding. The binding site in the duplex near the tethering end has d(5′-AAGAA-3′)/d(3′-TTCTT-5′) sequence in the 14 triplets long triple helix region and it can be d(5′-TTAAGAA-3′)/d(3′-AATTCTT-5′) if the base pairs outside the triplex region were also considered. The minor groove binding bisbenzimidazole can bind with both of these sites and the linker used in the present study is comparable with the hexaethyleneglycol linker in length used in a previous study[25] and it provides the sufficient flexibility to the bisbenzimidazole moiety to bind with this sequence. The bisbenzimidazole remains bound with the duplex DNA after the opening of triple helix and increases the melting temperature of duplex to coil transition.

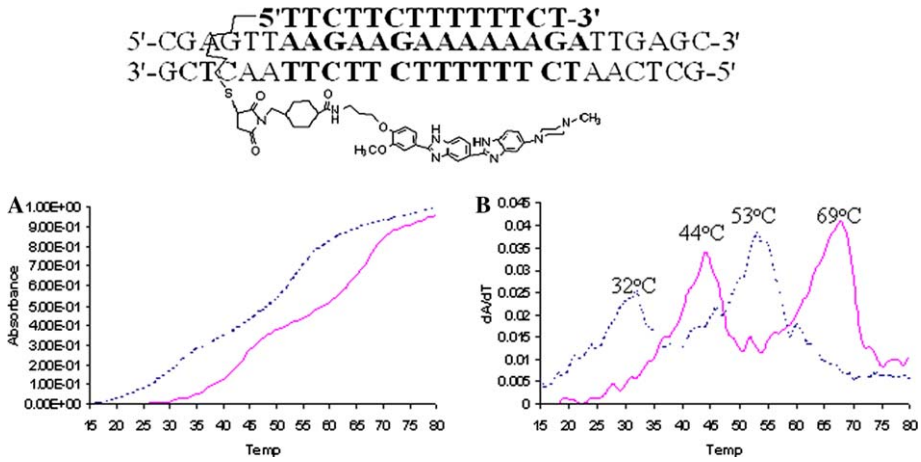


Figure 3. Absorbance versus temperature curves of triple helices with unconjugated (broken lines) and conjugated (solid lines) TFO (A) and their first derivatives (B). The triplex concn was 1 μM and the buffer used was 20 mM sodium cacodylate having 100 mM NaCl, pH 5.2.

2.4. Reduced emission intensities with increasing temperature

The free ligand used in this study ($BBZPNH_2$) has λ_{ex} = 350 nm and λ_{em} = 490 nm and DNA bound ligand has fluorescent maxima at 355 nm and 474 nm, respectively. The fluorescence emission of $BBZPNH_2$–maleimide–DNA conjugate enhanced many fold upon binding to target dsDNA. On excitation at 355 nm the triple helix made from $BBZPNH_2$–maleimide–DNA conjugate and dsDNA emits broad signal centered at 474 nm. To determine the fluorescence properties of the triple helix with conjugated TFO, the triple helix solution (100 nM) was excited at 355 nm and emission spectra were taken at different temperatures (5 °C increments). There is a progressive decline in the intensity of fluorescence signal with increase in temperature (Fig. 4A). There is only a marginal decrease in the fluorescence intensity after 44 °C, the temperature at which triplex to duplex transition occurs, that is, $BBZPNH_2$ remains bound with DNA duplex after the unwinding of the third strand from the major groove. There is a sharp decrease at 70 °C, that is, near the duplex to coil transition temperature clearly indicating bisbenzimidazole is free. From 20 °C to 70 °C the λ_{em} value is 474 nm, but after 70 °C this value increases to 490 nm that is the value for free ligand. This is a clear indication that the bisbenzimidazole remains bound with the duplex DNA up to 70 °C.

2.5. Induced ellipticity with increasing temperature

Both double and triple helical DNA does not have any CD signal after 300 nm and being achiral molecule, bisbenzimidazole dyes (e.g., Hoechst 33258) do not show any CD signal. After binding with DNA, $BBZPNH_2$ show, induced signal near 375 nm (for Hoechst 33258 it is near 356 nm).[18] Ellipticity versus wavelength plot of 5 μM solution of triple helix formed by bisbenzimidazole–maleimide–DNA conjugate and 26 bp long DNA duplex at the increment of 5 °C clearly indicates an induced CD signal up to 70 °C (Fig.4B). There is a large decrease in the intensity of the CD signal at 75 °C, which clearly indicates that the bisbenzimidazole

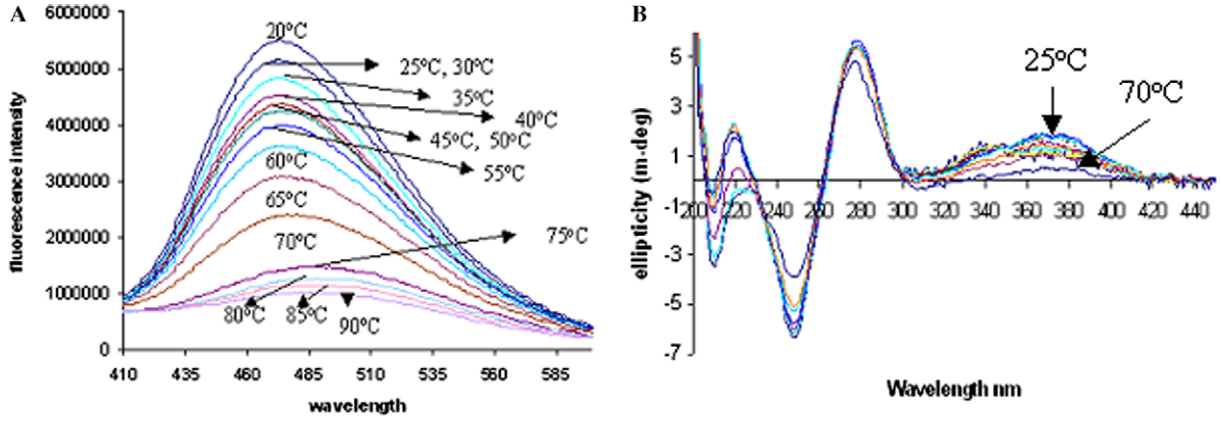


Figure 4. Change in fluorescence emission intensity with temperature of a 100 nM solution of triple helix (formed from $BBZPNH_2$–maleimide–DNA conjugate and 26mer dsDNA) (A) and Change in circular dichroism spectrum with temperature of a 3 μM solution of triple helix (B). The buffer used was 20 mM sodium cacodylate having 100 mM NaCl, pH 5.2.

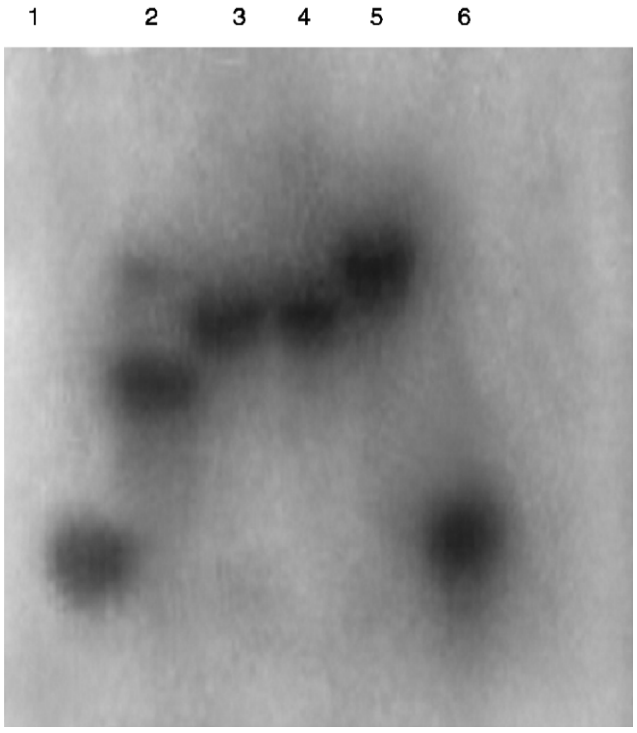


Figure 5. EMSA of triple helix. Gel was run in 50 mM Tris–acetate having 10 mM $MgCl_2$, pH 5.2, using 10% polyacrylamide gel, at 10 °C. Lanes 1 and 6, 26mer single strand CGAGTTA AGAAGAAAAAAG-ATTGAGC (oligo-3); lane 2, 26 bp long target duplex; d(CGAGTT AAGAAGAAAAAAGATTGAGC); lanes 3 and 4, triple helix made from non-conjugated TFO TTCTTCTTTTTTCT (oligo-2) and duplex d(CGAGTTAAGAAGA AAAAA GAT T GAGC); lane 5, triple helix made from conjugated TFO and duplex d(CGAGTTAAG AAGAAAAAAGATTGAGC).

moiety remains bound in the minor groove of duplex DNA even after third strand separates from the major groove (T_m = 44 °C).

2.6. Gel mobility shifts

The triple helix formed with conjugated TFO has retarded mobility than that formed with the normal TFO (Fig. 5). Both triplexes have C^+GC triplets and the net negative charge of the helices is reduced and both the triplexes have shown the retarded mobility. The $BBZPNH_2$ moiety present in conjugated TFO becomes protonated at pH 5.2, hence there is further reduction in the negative charge of the triple helix formed with conjugated TFO and hence triple helix has the lower mobility than the second triple helix.

3. Conclusions

An analogue of Hoechst 33258 having bisubstitution on the terminal phenyl ring ($BBZPNH_2$) has been synthesized and reacted successfully with the free thiol group present at 5′-end of synthetic 14mer DNA sequence with the help of SMCC, a heterobifunctional crosslinking reagent. The synthesis of the conjugate is simple and extremely anhydrous conditions required in the phosphoramidite chemistry are no longer required in the present approach as aqueous solutions were used in the synthesis. This conjugate was hybridized successfully with the 26 base pairs long target duplex. The 14mer DNA sequence of the conjugate binds in the major groove of dsDNA and the dye $BBZPNH_2$ binds in the AT rich minor groove of the target duplex. The conjugation enhances the stability of $BBZPNH_2$–maleimide–DNA conjugate–$(DNA)_2$ triple helix through simultaneous major and minor groove binding. $BBZPNH_2$ remains bound in the minor groove of dsDNA even after the separation of 14mer TFO from the major groove. The 26 atom long linker present between DNA sequence and dye provides sufficient flexibility to the dye to bind in the minor groove of the target duplex. The conjugate has shown enhanced cellular and nuclear membrane permeability due to the bisbenzimidazole dye covalently linked to SMCC. Cellular uptake experiment with U87 cells suggests that $BBZPNH_2$–maleimide–DNA conjugate has comparable cell permeability with Hoechst 33342, $BBZPNH_2$, and $BBZPNH_2$–maleimide.

4. Experimental

4.1. Materials

All solvents and reagents were obtained from Sigma–Aldrich Chemical Company, USA, and were used without further purification. TLCs were carried out on commercially available TLC silica gel (Silica gel 60 F254) plates purchased from E Merck, Germany, and compounds on TLC were visualized using short-wavelength UV light. Silica gel (70–220 mesh size, E Merck, Germany) was used for flash column chromatography. The purity of all organic compounds was confirmed by TLC, NMR, IR, etc. IR were recorded on FTIR 8300 Shimadzu Spectrophotometer. NMR spectra were recorded on Bruker 300 MHz NMR Instrument. Melting points were taken on a Buchi Melting point B540 apparatus. Elemental analysis was done on GmbH VarioeEL elemental analyser. The FAB Mass spectra were recorded on JEOL SX 102/DA-6000 Mass spectrometer using *m*-nitrobenzyl alcohol (NBA) as a matrix. The MALDI mass spectra were obtained with a MALDI Kratos Analytical Kompact SEQ IV, UK, mass spectrometer using α-cyano-4-hydroxycinnamic acid as a matrix. ESMS analysis was performed on a Hewlett-Packard 1100 MSD electrospray mass spectrometer. Samples for ESMS were prepared in 50% acetonitrile/water having 1% triethylamine and sample concentrations were 20–50 pM. Deionized and nuclease, free water was used to prepare all the buffers. PAGE, purified oligonucleotides and 14mer HPLC purified oligonucleotide sequence having free thiol group at 5′-end linker were purchased from Microsynth, Switzerland. Succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) was purchased from Pierce. NAP-10 (Sephadex G-25 DNA grade) columns were purchased from Amersham Biosciences. HPLC grade acetonitrile and water were purchased from J.T. Baker, and triethylammonium acetate (TEAA) was purchased from Sigma–Aldrich

Chemical Company, USA. Reverse-phase HPLC was performed on a LC-8A Shimadzu instrument equipped with a quaternary solvent delivery system and a diode array detector. YMC-Pack ODS-AQ (250 × 4.6 mm) Reverse-phase column was used. UV detector was set at 260 nm for DNA, and 260 and 343 nm for $BBZPNH_2$–malemide–DNA conjugate. A gradient from 5% to 30% of eluent B (50 mM TEAA buffer, pH 7, containing 50% acetonitrile) eluent A (50 mM TEAA buffer, pH 7) over 30 min with a flow rate of 1.0 mL/min was used. The extinction coefficients of DNA were determined according to the nearest neighbor method at 260 nm and for the conjugate the extinction coefficient of the $BBZPNH_2$ at 260 nm (16,000 U mM^{-1}) was added to the calculated value of DNA. However, the concentrations of conjugate solutions were determined using the extinction coefficient (25,000 ε_{345} U M^{-1}) of $BBZPNH_2$.

4.2. Thermal melting studies

All T_m experiments were carried out in 20 mM sodium cacodylate buffer having 100 mM NaCl at pH 5.2 having oligomer concentrations of 1 μM for each strand. The oligonucleotide solutions were annealed by heating to 75 °C using a heating block and allowing to cool slowly to reach room temperature before being stored at 4 °C. Absorbance (260 nm) versus temperature values were obtained on a Cary Varian 400 spectrophotometer equipped with a temperature programmable cell block using 1 cm path length quartz cell. Data points between 15 and 85 °C were taken for every 1 °C, with a temperature ramp of 0.25 °C/min. T_m values were calculated by first-derivative analysis and also by direct graphical analysis of the absorbance versus temperature plot to determine the midpoint of the transition. Both techniques gave values that were within the experimental error (1 °C) for the analysis. The concentration-dependent T_m study was also carried out to rule out the possibility of intramolecular duplex formation.

4.3. Fluorescence spectroscopy

Fluorescence spectra were obtained on a Horiba, Jovin Yvon FloroMax-3 spectrometer equipped with a thermal programmer. All measurements were performed with the following parameters: slit width, Ex/Em = 10 nm/10 nm; high sensitivity; high speed (500 nm/s). The components of triple helix were annealed in sodium cacodylate buffer (20 mM sodium cacodylate containing 100 mM NaCl, pH 5.2), prior to use, and solutions were introduced into 2.5 mL quartz cell thermally insulated with a water jacket. The concentration of solutions was 100 nM. The solutions were excited at 355 nm, and emissions were monitored between 360 and 600 nm at different temperatures.

4.4. Circular dichroism spectroscopy

CD spectroscopy experiments were done on a Jasco-800 CD spectropolarimeter equipped with a temperature programmable cell block in a 2 mm path length quartz cell. To make different DNA structures (WC duplex and DNA triple helix), the complementary oligonucleotide strands were annealed in sodium cacodylate buffer (20 mM sodium cacodylate containing 100 mM NaCl, pH 5.2), prior to use. Spectra were scanned from 200 to 450 nm at different temperatures with a scan speed of 50 nm/min.

4.5. Electrophoretic mobility shift assay

Nondenaturing PAGE is used for the separation and purification of double stranded DNA. Oligonucleotides were 5′-end labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase (MBI Fermentas) using the standard protocol given by the supplier, purified by phenol–chloroform extraction and ethanol precipitation, and then made free from unincorporated [γ-^{32}P] ATP by Sephadex G-25 according to the manual instructions. The labeled oligonucleotide was hybridized with equimolar amount of complementary strand to make the duplex. Triplex formation was initiated by adding 3 μL of 3× buffer (135 nM Tris–acetate, pH 5.2, having 30 mM $MgCl_2$), 1.2 equiv of conjugated or non-conjugated TFO, and 1 equivalent of labeled duplex to a final 10 μL reaction volume. Solutions were heated to 75 °C in a heating block then cooled slowly to room temperature followed by addition of 3 μL 50% glycerol solution containing orange-G. The samples were directly loaded onto a 10% native polyacrylamide gel prepared in buffer (50 mM Tris–acetate, pH 5.2, and 10 mM $MgCl_2$). Electrophoresis was performed at 8 V/cm for 10 h at 10 °C in buffer (50 mM Tris–acetate, pH 5.2 and 10 mM $MgCl_2$). The dried gels were exposed to Kodak film at −80 °C and then developed.

4.6. Synthesis of 3-*N*-(*tert*-butyloxycarbonyl)-propan-1-ol (1)

Di-*tert*-butyl dicarbonate (4.36 g, 20 mmol) dissolved in dioxane (30 mL) was added dropwise to a solution of 3-amino propanol (1.5 g, 20 mmol) in dioxane (70 mL) with stirring at room temperature. Reaction mixture was stirred for about 10 h and then dioxane was removed under reduced pressure. Residue was dissolved in water and the product was extracted with ethyl acetate. Organic phase was evaporated to give yellowish oil. Yield: 90%. IR: 3336, 2977, 2936, 1690, 1525, 1455, 1367, 1171 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 1.45, (s, 9H), 1.8 (m, 2H), 2.9 (t, 2H), 3.5 (t, 3H).

4.7. Synthesis of 4-[3-*N*-(*tert*-butyloxycarbonyl)-propyloxy]-5-methoxybenzaldehyde (3)

4-Hydroxy-3-methoxy benzaldehyde (1.21 g, 8 mmol) and alcohol (**X**) (1.272 g, 8 mmol) were taken in a three-necked RB flask in DCM (40 mL). Triphenylphosphine (3.4 g, 13.1 mmol) and diethylazodicarboxylate (DEAD) (2.3 g, 13.1 mmol) were added to it. Reaction mixture was stirred under nitrogen at 0 °C for 30 min and then overnight at room temperature. Reaction mixture was then concentrated to get an oily residue. This oil was added to 50% ethyl acetate–petroleum ether to get solid oxytriphenylphosphine, which was filtered out. Filtrate was concentrated and chro-

matographed on silica gel using benzene–ethyl acetate (10–12%) as eluent. Product was obtained as white crystalline solid. Yield: 60%; mp: 66–70 °C. IR: 3368, 2980, 2937, 1683.5, 1593.6, 1526 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 1.46 (s, 9 H), 2.04 (m, 2H), 3.94 (s, 3H), 4.18 (d, 2H), 6.94 (d, 1H), 7.5 (d, 1H), 9.85 (s, 1H); *m/z* (FAB mass) found 310 (M+1), calculated 309.

4.8. Synthesis of 5-(4-methylpiperazin-1-yl)-2-[2′-(4-{3-aminopropyloxy}-3-methoxyphenyl)-5′-benzimidazolyl]-benzimidazole (6)

Water (around 1 mL) and $Na_2S_2O_5$ (141 mg) were added to a solution of 2-amino-4-[5′-(4″-methylpiperazin-1″-yl)benzimidazol-2′-yl]aniline (**4**) and aldehyde (**3**) (455 mg, 1.5 mmol) in ethanol (100 mL) and reaction mixture was refluxed for 7 h, cooled, and filtered through Celite. Filtrate was concentrated and purified on silica column using methanol–ethyl acetate as eluent to give **5**. Mp: 265–270 °C; Yield: 60%. IR: 3401, 2922, 1683, 1635, 1497, 1446, 1371, 1281, 1131, 819 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6)δ ppm: 1.45 (s, 9H), 2.05 (m, 2H), 2.33 (s, 3H), 3.5 (t, 4H, J = 4.8 Hz), 3.98 (s, 3H), 4.12 (d, 2H, J = 7 Hz), 6.77 (d, 1H, J = 9.0 Hz), 7.13 (d, 2H, J = 9.0 Hz), 7.64 (d, 1H, J = 9.0 Hz), 7.81 (d, 1H, J = 9.0 Hz), 8.03 (m, 1H), 8.17 (d, 2H, J = 9.0 Hz), 8.3 (s, 1H), 13.0 (br s, 2H); ^{13}C NMR (70 MHz, DMSO-d_6) δ ppm: 28.7, 32.0, 40.9, 42.7, 59.7, 70.8, 100.0, 113.8, 115.7, 116.0, 120.0, 116.3, 121.4, 127.5, 129.1, 139.0, 141.6, 144.6, 148.3, 158.0; *m/z* (FAB mass) found 612 (M+1), calculated 611. Anal. Calcd for $C_{34}H_{41}N_7O_4$: C, 66.78; H, 6.71; N, 16.04. Found: C, 66.75; H, 6.70; N, 16.06. Compound **5** (350 mg) was dissolved in 2 N HCl (80 ml) and refluxed for 10 min, cooled and evaporated under reduced pressure, and then purified on silica gel column to give **6**. Yield: 67%; mp: 280 °C; IR: 3401, 2922, 1635, 1497, 1446, 1371, 1281, 1131, 819 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.90 (m, 2H), 2.33 (s, 3H), 3.5 (t, 4H), 3.98 (t, 2H), 4.04 (s, 3H), 6.77 (d, 1H, J = 9.0 Hz), 7.13 (d, 2H, J = 9.0 Hz), 7.64 (d, 1H, J = 9.0 Hz), 7.81 (d, 1H, J = 9.0 Hz), 8.03 (m, 1H), 8.17 (d, 2H, J = 9.0 Hz), 8.3 (s, 1H), 13.0 (br s, 2H); ^{13}C NMR (70 MHz, DMSO-d_6) δ ppm: 34.8, 40.9, 42.7, 59.7, 100.0, 113.8, 115.7, 116.0, 120.0, 116.3, 121.4, 127.5, 129.1, 139.0, 141.6, 144.6, 148.3; *m/z* (FAB mass) found 512 (M+1), calculated 511. Anal. Calcd for $C_{29}H_{33}N_7O_2$: C, 68.10; H, 6.46; N, 19.18. Found: C, 68.15; H, 6.42; N, 19.15.

4.9. Introduction of maleimide moiety at the free NH_2 group of $BBZPNH_2$

A solution of bisbenzimidazole 5-(4-methylpiperazin-1-yl)-2-[2′-(4-{3-aminopropyloxy}-3-methoxyphenyl)-5′-benzimidazolyl] benzimidazole in DMF (60 μL of 10 mM solution, 60 nmol) was taken in the reaction tube and volume was increased to 200 μL by DMF. Now SMCC (3 μmol, dissolved in 20 μL DMF) was added to this solution followed by addition of DIEA (20 μL). The reaction solution was left at room temperature for half an hour with gentle shaking. Now cold dry diethyl ether (about 10 mL) was added to the reaction solution to precipitate the $BBZPNH_2$–maleimide conjugate. This precipitate was washed five times with cold diethyl ether containing 10% *N*-methyl morpholine (NMP) to remove excess SMCC and dried under N_2. The product was characterized by ESMS in positive ion mode *m/z* found 731.23 (M+1), calculated: 730. ^1H NMR (DMSO-d_6) δ ppm: 1.42 (t, 4H, J = 4.8 Hz), 1.77 (t, 4H, J = 5.0 Hz), 1.80 (m, 2H), 2.10 (t, 1H, J = 2.5 Hz), 2.33 (s, 3H), 2.4 (d, 1H, J = 5.0 Hz), 3.5 (t, 4H, J = 4.8 Hz), 3.98 (t, 2H, J = 5.0 Hz), 4.04 (s, 3H), 6.77 (d, 1H, J = 9.0 Hz), 7.0 (m, 2H), 7.13 (d, 2H, J = 9.0 Hz), 7.64 (d, 1H, J = 9.0 Hz), 7.81 (d, 1H, J = 9.0 Hz), 8.03 (m, 1H), 8.17 (d, 2H, J = 9.0 Hz), 8.3 (s, 1H), 13.0 (br s, 2H); ^{13}C NMR (70 MHz, DMSO-d_6) δ ppm: 31.1, 25.0, 25.8, 32.8, 40.9, 42.7, 45.0, 59.7, 100.0, 113.8, 115.7, 116.0, 120.0, 116.3, 121.4, 127.5, 129.1, 137.0, 139.0, 141.6, 144.6, 148.3; 165.0, 179.0.

4.10. Synthesis of $BBZPNH_2$–maleimide–DNA conjugate (9)

Bisbenzimidazole–maleimide conjugate was dissolved in 200 μL DMF and a solution of 14mer oligonucleotide in water having free 5′-SH group (500 μL of 100 μM solution, freshly made from lyophilized oligonucleotides, total 50 nmol) was added to this solution. The pH of reaction mixture was adjusted at 7.8 by adding triethylamine and left for overnight shaking. The reaction mixture was then loaded on a Sephadex G-25 column pre-equilibrated with water and the required bisbenzimidazole–maleimide–DNA conjugate free from unincorporated bisbenzimidazole–SMCC was eluted under gravity. The product was lyophilized and made up to 100 μL by water and purified by reverse phase HPLC. The product peak was collected and quantitated by absorbance at 345 nm (A_{345}) and lyophilized (35 nmol, 70% isolated yield). The purity of the product was confirmed by reinjecting an analytical amount of the conjugate on a reverse phase HPLC column.

Analytical reverse phase HPLC t_R, 25.02 min (for 14mer oligonucleotide having 5′-free thiol t_R, 11.64 min); *m/z* (negative mode ESMS) mass found 5077.45, calculated 5077.5.

Acknowledgment

A.K.J. is thankful to Council for Scientific and Industrial Research (CSIR), New Delhi, for financial support as SRF.

Supplementary data

UV spectra, mass spectra of H-SMCC Conjugate, and cellular uptake by U87 cells of the conjugate are given in supporting information. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.05.034.

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