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Synthesis and biological activities of bisnaphthalimido polyamines derivatives: cytotoxicity, DNA binding, DNA damage and drug localization in breast cancer MCF 7 cells

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Abstract

New bisoxynaphthalimidopolyamines (BNIPOPut, BNIPOSpd and BNIPOSpm) were synthesized. Their cytotoxic properties were evaluated against breast cancer MCF 7 cells and compared with bisnaphthalimidopolyamines BNIPSpd and BNIPSpm. Among the bisoxynaphthalimido polyamines, BNIPOSpm and BNIPOSpd exhibited cytotoxic activity with an IC_{50} of 29.55 and 27.22 μM, respectively, while BNIPOPut failed to exert significant cytotoxicity after 48-h drug exposure. DNA binding was determined by midpoint of thermal denaturation (T_m) measurement, ethidium bromide displacement and DNA gel mobility. Both BNIPOSpm and BNIPOSpd exhibited strong binding affinities with DNA. BNIPOPut had the least effect. The results were compared with other cytotoxic bisnaphthalimido compounds (BNIPSpm and BNIPSpd) previously reported by us. Using the single cell gel electrophoresis assay, it was found that BNIPSpm and BNIPSpd caused substantial DNA damage to MCF 7 treated cells while BNIPOSpm showed no significant effect over a range of drug concentrations after 4-h drug exposure. However, after 12-h drug exposure, BNIPOSpm had induced significant DNA damage similar to that of BNIPSpm (after 4-h drug exposure). Fluorescence microscopic analysis revealed that at 1 μM drug concentration and after 6-h drug exposure, both BNIPSpm and BNIPSpd were located within the cell while the presence of BNIPOSpm, was not observed. Therefore, we conclude that BNIPSpd, BNIPSpm and BNIPOSpm induce DNA damage consistent with their rate of uptake into the cells.

Keywords: Bisnaphthalimides; Synthesis; Cytotoxicity; DNA binding and damage; Single cell gel electrophoresis assay; Fluorescence microscopy

1. Introduction

Studies on molecules interacting with DNA are rapidly generating intense interest in many research laboratories [1,2]. Some of them have found applications as anticancer agents [3], as important dyes used in molecular biology [4] and as tools to understand a number of biological processes [2].

Naphthalimides and bisnaphthalimides are well-known cytotoxic DNA intercalating agents and have shown promise as potential anti-cancer agents [5]. Recently the bisnaphthalimide, elinafide (Fig. 1) has gone into phase I clinical trials in Europe and the USA [6]. NMR spectroscopy, suggests that elinafide bisintercalates to specific DNA sequence with the propylene diamine linker lying in the major groove of the DNA molecule [7]. Although bisnaphthalimides have shown to have higher anticancer activity than the mononaphthalimides, they are however very insoluble and can render testing difficult [8].

In a previous report, we described the synthesis and cytotoxic properties of bisaminopropylnaphthalimido

Abbreviations: BNIPPut, bisnaphthalimidopropylputrescine; BNIPSpd, bisnaphthalimidopropylspermidine; BNIPSpm, bisnaphthalimidopropylspermine; BNIPOPut, bisnaphthalimidooxypropylputrescine; BNIPOSpd, bisnaphthalimidooxypropylspermidine; BNIPOSpm, bisnaphthalimidooxypropylspermine; DMF, dimethylformamide; THF, tetrahydrofuran; MTT, 3-(4,5-diemthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; T_m, midpoint of thermal denaturation

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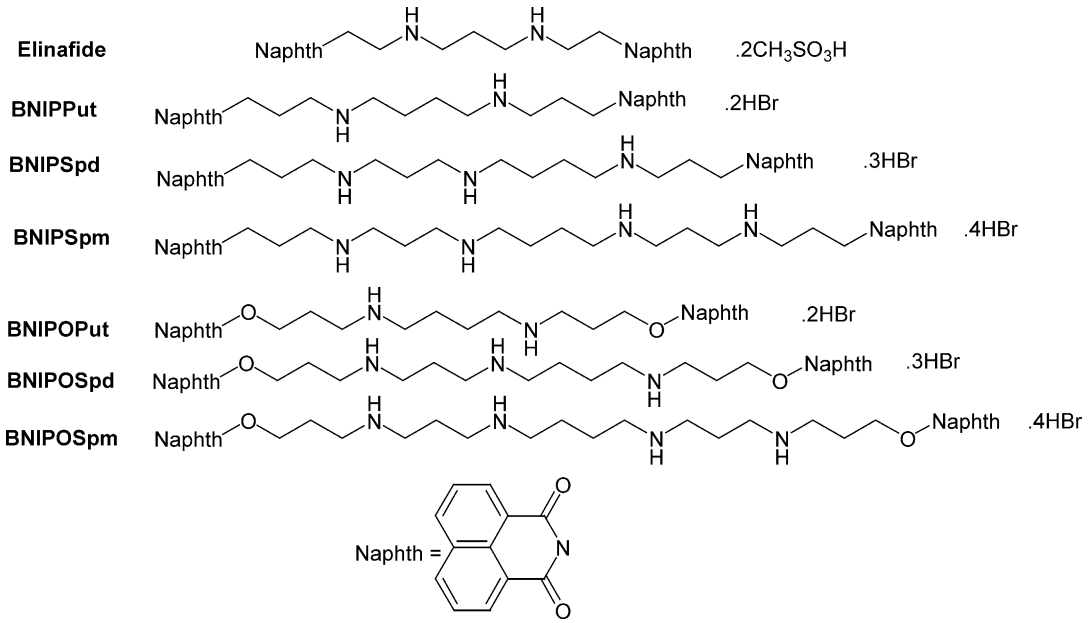

Fig. 1. Chemical structures of bisnaphthalimides.

polyamines (BNIPSpd, BNIPSpm in Fig. 1) containing more than two nitrogen atoms in the linker chain with improved solubility without unduly compromising their biological activity [9,10]. We reason that the higher the number of heteroatoms present in the linker chain, the higher the solubility of these compounds. Furthermore, they strongly stabilized DNA duplexes and were localized preferentially inside the nucleus [10]. Previous studies in our laboratory demonstrated that compound BNIPSpd might take part in the initiation of apoptosis in MCF 7 breast and HL60 Leukemia cancer cells [11,12].

In this paper, we have extended our previous work [9,12] by synthesizing three new bisaminooxypropylnaphthalimido polyamines derivatives, BNIPOPut, BNIPOSpd and BNIPOSpm (Fig. 1) in which oxygen atoms are introduced in the α-position with respect to the naphthalimido ring. The rationale in synthesising BNIPOPut, BNIPOSpd and BNIPOSpm is to show that such modification would enhance the solubility of those compounds in aqueous media without affecting their biological activity. Their cytotoxicities against MCF 7 cells were evaluated and their DNA binding affinities were studied using UV, fluorescence and gel mobility measurements. The results obtained were compared with BNIPSpd and BNIPSpm (Fig. 1). BNIPPut containing two nitrogen atoms in its linker chain could not be used in our study because of its high insolubility. We have also for the first time studied the extent of DNA damage induced by BNIPSpd, BNIPSpm and BNIPOSpm in drug treated MCF 7. Using fluorescence microscopy, we found previously that BNIPSpd was located in the cell nucleus after 8-h drug exposure [10]. In this paper, we have also extended the microscopic studies with MCF 7 cells exposed to BNIPSpd, BNIPSpm and BNIPOSpm over differing time scales in order to relate the timing of uptake and localisation to the extent of DNA damage and cytotoxicity.

2. Materials and methods

2.1. Materials

Cell lines were obtained from ECACC. All reagents were purchased from Aldrich, Fluka and Lancaster and were used without purification. TLC was performed on Kieselgel plates (Merck) 60 F_{254} in chloroform: methanol (97:3 or 99:1). Column chromatography was done with silica gel 60, 230–400 meshes using chloroform and methanol as eluent. FAB-mass spectra were obtained on a VG Analytical AutoSpec (25 kV) spectrometer; EC/CI spectra were performed on a Micromass Quatro II (low resolution) or a VG Analytical ZAB-E instrument (accurate mass). ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-EX90 FT NMR spectrometer.

BNIPPut, BNIPSpd and BNIPSpm were synthesised according to our methods previously reported [9,13].

2.2. Synthesis of 3-phthalimidooxy-1-propanol, **2**

N-hydroxyphthalimide (9.2 g, 0.0565 mol) was dissolved in DMF (50 mL) followed by the addition of 3-bromopropanol (7 g, 0.0504 mol) and triethylamine (5.7 g, 0.565 mol). The solution was left overnight at 85 °C. The solvent was removed under vacuo and the residue dissolved in chloroform (50 mL). The organic phase was washed with (i) water and (ii) saturated bicarbonate solution. The chloroform layer was dried with anhydrous sodium sulphate and removal of solvent gave **2** (7.91 g,

71%) as a waxy solid. ^{1}H NMR ($CDCl_3$): δ = 7.70–8.00 (m, 4H, phthalimido protons), 4.50 (t, 2H, O–CH_2), 4.10 (t, 2H, –CH_2–O), 2.50 (broad, 1H, OH), 2.10 (p, 2H, 2H, –CH_2–). ^{13}C NMR ($CDCl_3$): δ = 163.94 (C=O), 134.74, 129.15, 123.78 (aromatic C), 75.78 (OCH_2), 59.28 (CH_2O), 31.10 (CH_2). Compound **2** was used in the next step without further purification.

2.3. Synthesis of 3-aminooxy-1-propanol, 3

Compound **2** (7.91 g, 0.0358 mol) was dissolved in absolute ethanol followed by the addition of hydrazine hydrate (1.32 g, 41.2 mmol). The solution was left stirring at room temperature overnight. The resulting white precipitate was filtered off. The filtrate was evaporated to dryness and the residue was resuspended in dichloromethane (25 mL). The solution was filtered and the filtrate was reduced under vacuo to give **3** (2.33 g, 64.4%) as a thick oil. ^{1}H NMR ($CDCl_3$): δ = 4.20 (s, broad, 2H, ONH_2), 3.50–4.00 (m, 4H, OCH_2, CH_2O), 1.70–2.00 (p, 2H, –CH_2–). ^{13}C NMR ($CDCl_3$): δ = 75.00 (OCH_2), 61.78 (CH_2O), 32.71 (–CH_2–). LRMS (FAB): Calcd. for $C_3H_9NO_2$ 91, found: 92 [*M*H]$^+$.

2.4. Synthesis of 3-(N-naphthalimidooxy)-propan-1-ol, 4

Naphthalic anhydride (6.34 g, 0.032 mol) was dissolved in DMF (50 mL) followed by the addition of aminooxypropanol **3** (2.33 g, 0.0256 mol) and DBU (4.87 g, 0.032 mol). The solution was left stirring at 85 °C. The solvent DMF was removed under reduced pressure and the resulting residue was dissolved in dichloromethane (100 mL). The organic layer was washed with (i) water and (ii) saturated bicarbonate solution. After drying with anhydrous sodium sulphate and removal of the solvent, gave a crude solid, which was subjected to column chromatography to give pure product **4** as a white powder (1.97 g, 26.4%). NMR ($CDCl_3$): δ = 8.65–7.80 (m, 6H, aromatic protons), 4.45 (t, 2H, –N–OCH_2), 4.05 (t, 2H, CH_2–O–), 3.40 (s, broad, 1H, OH), 2.10 (p, 2H, CH_2). ^{13}C NMR ($CDCl_3$): δ = 161.70 (C=O), 135.70–122.90 (aromatic carbons), 74.90, 59.90, 30.90 (3× CH_2). LRMS (FAB): Calcd. for $C_{15}H_{13}NO_4$ 271.08, found: 272 [*M*H]$^+$.

2.5. Synthesis of O-tosylpropyloxynaphthalimide, 5

Compound **4** (1.97 g, 7.26 mmol) was dissolved in anhydrous CH_2Cl_2 (8 mL) followed by the addition of tosyl chloride (6.09 g, 0.032 mol) and triethylamine (4.0 mL). The solution was left overnight at 50 °C. After cooling the CH_2Cl_2 solution was washed with (i) water and (ii) saturated bicarbonate solution. After drying and the removal of the solvent, a dark residue was obtained which was then subjected to column chromatography. The main fraction gave **5** (1.02 g, 33%) as a buff solid. ^{1}H NMR ($CDCl_3$): δ = 8.70–7.35 (m, 6H, aromatic protons), 4.45 (t, 2H, CH_2), 4.35 (t, 2H, CH_2), 2.50 (s, 3H, CH_3), 2.25 (p, 2H, CH_2). ^{13}C NMR ($CDCl_3$): δ = 161.30 (C=O), 145.10–123.10 (aromatic carbons), 73.10, 67.90, 28.70 (3× CH_2), 22.10 (CH_3). LRMS (FAB): Calcd. for $C_{12}H_{19}NO_6S$ 425.09, found: 426 [*M*H]$^+$.

2.6. General N-alkylation reaction (Fig. 2)

Mesitylated polyamines [9,12] (0.651 mmol) were dissolved in anhydrous DMF (13.5 mL) followed by the addition of **5** (0.13 mmol) and cesium carbonate (1.06 g). The solution was left at 85 °C and completion of the reaction was monitored by thin layer chromatography. DMF was removed under vacuo and the residue was dissolved in chloroform and extracted with (i) water and (ii) saturated bicarbonate solution. The crude product was purified by column chromatography.

2.7. General deprotection reaction (Fig. 2)

The fully protected polyamine derivatives (0.222 mmol) were dissolved in anhydrous dichloromethane (10 mL) followed by the addition of hydrobromic acid/glacial acetic acid (1 mL). The solution was left stirring at room temperature for 24 h. The yellow precipitate formed, was filtered off and washed with dichloromethane, ethylacetate and ether.

2.8. Synthesis of BNIPOPut

The reaction between *N*,*N*-dimesitylputrescine **6** with *O*-tosylpropyloxynaphthalimide **5** gave BNIPOPut as yellow solid (48% yield). ^{1}H NMR (DMSO-d_6): δ = 8.62 (s, broad, –$^+NH_2$), 8.43–8.36 (m, aromatic hydrogens), 7.82–7.77 (m, aromatic hydrogens), 4.29–4.25 (t, O–CH_2–), 3.27–3.25 (m, N–CH_2–), 3.10 (m, broad, N–CH_2–), 2.10–2.07 (m, –CH_2–), 1.79 (m, broad, –CH_2–).^{13}C NMR (DMSO-d_6): δ = 160.71 (C=O), 135.21–121.83 (aromatic carbons), 75.00–22.00 (10× CH_2). LRMS (FAB): Calcd. for $C_{34}H_{34}N_4O_6$ 2HBr 756.66, found: 426 [*M*–2HBr]$^+$.

2.9. Synthesis of BNIPOSpd

The reaction between *N*,*N*,*N*-trimesitylspermidine **7** with *O*-tosylpropyloxynaphthalimide **5** gave BNIPOSpd as yellow solid (72% yield). ^{1}H NMR (DMSO-d_6): δ = 8.72 (s, broad, –$^+NH_2$), 8.62 (s, broad, –$^+NH_2$), 8.58–8.50 (m, aromatic hydrogens), 7.95–7.85 (m, aromatic hydrogens), 4.35–4.25 (m, O–CH_2–), 3.35–3.25 (m, N–CH_2–), 3.15–2.90 (m, N–CH_2–), 2.20–2.00 (m, –CH_2–). ^{13}C NMR (DMSO-d_6): δ = 161.00 (C=O), 135.50–122.80 (aromatic carbons), 74.20–22.70 (13× CH_2). LRMS (FAB): Calcd. for $C_{37}H_{41}N_5O_6$ 3HBr 894.32, found: 654 [*M*–3HBr]$^+$.

2.10. Synthesis of BNIPOSpm

The reaction between *N*,*N*,*N*,*N*-tetramesitylspermine **8** with *O*-tosylpropyloxynaphthalimide **5** gave BNIPOSpm as yellow solid (68% yield). ^{1}H NMR (DMSO-d_6): δ = 8.64 (s, broad, $-^+NH_2$), 8.48–8.36 (m, aromatic hydrogens), 7.82–7.77 (m, aromatic hydrogens), 4.29 (m, O–CH_2–), 3.27 (m, N–CH_2–), 3.10 (m, N–CH_2–), 2.10–2.07 (m, –CH_2–), 1.79 (m, –CH_2–). ^{13}C NMR (DMSO-d_6): δ = 161.07 (C=O), 135.58–122.20 (aromatic carbons), 74.20–23.12 (16× CH_2). LRMS (FAB): Calcd. for $C_{40}H_{48}N_6O_6$ 4HBr 1032.36, found: 709 [*M*–4HBr]$^+$.

2.11. Cytotoxic studies-MTT assay

MCF 7 cells were maintained in Earle's minimum essential medium (Sigma), supplemented with 10% (v/v) foetal calf serum (Labtech Int.), 2 mM L-glutamine (Sigma), 1% (v/v) non-essential amino acids (Sigma), 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Sigma). Exponentially growing cells were plated at 2×10^4 cells cm^{-2} into 96-well plates and incubated for 24 h before the addition of drugs. Stock solutions of compounds were initially dissolved in 20% (v/v) DMSO and further diluted with fresh complete medium.

The growth-inhibitory effects of the compounds were measured using standard tetrazolium MTT assay [15]. After 24 and 48 h of incubation at 37 °C, the medium was removed and 200 μL of MTT reagent (1 mg/mL) in serum free medium was added to each well. The plates were incubated at 37 °C for 4 h. At the end of the incubation period, the medium was removed and pure DMSO (200 μL) was added to each well. The metabolized MTT product dissolved in DMSO was quantified by reading the absorbance at 560 nm on a micro plate reader (Dynex Technologies, USA). IC_{50} values are defined, as the drug concentrations required to reduce the absorbance by 50% of the control values. The IC_{50} values were calculated from the equation of the logarithmic line determined by fitting the best line (Microsoft Excel) to the curve formed from the data. The IC_{50} value was obtained from the equation for $y = 50$ (50% value).

2.12. Thermal denaturation studies

Optical thermal denaturation experiments were performed in stoppered quartz cuvettes using a Perkin-Elmer Lambda 2 UV–vis spectrophotometer fitted with a Peltier circulating heating/cooling temperature programmer and water supply. *Calf thymus* DNA (Sigma) working solutions (200 μM) were prepared by dissolving DNA (5 mg) in 100 mL of 0.01 M phosphate buffer (25 mM Na_2HPO_4, 15 mM NaH_2PO_4, and 1.25 mM EDTA). The DNA-drug solutions were prepared by the addition of the drug (20% DMSO/water) to give the desired final concentration. The binding affinity (i.e. T_m) was measured by determining the change in midpoint of the thermal denaturation curves of DNA.

2.13. Fluorescence-binding studies

Binding studies were carried out by competitive displacement fluorometric assay with DNA-bound ethidium. *Calf thymus* DNA (Sigma) was used without further purification. C_{50} values are defined as the drug concentration (μM), which generate a 50% decrease in the fluorescence of the bound ethidium/DNA.

Test solutions were made by adding 160 μL of compound in 0.01 M phosphate buffer (25 mM Na_2HPO_4, 15 mM NaH_2PO_4, and 1.25 mM EDTA) to a fixed volume of *calf thymus* DNA (20 μL of 16.6 μM) and EB (20 μL of 20 μM) in 96-microplates wells. All measurements were taken at room temperature using a Perkin-Elmer LS 55 luminescence spectrometer (excitation at 546 nm; emission at 595 nm).

2.14. Gel mobility assay

The interaction of the drugs with DNA was assessed on the basis of agarose gel mobility of the supercoiled plasmid pBR 322 (Biolabs Inc., N 3033 S). The plasmid (1 ng) was mixed with the polyamines derivatives (10 μM) and incubated at 37 °C for 30 min. Following the addition of the loading buffer (50% (v/v) glycerol, 0.25% w/v bromophenol blue), the sample was loaded onto 1% (w/v) agarose gel (made in TAE buffer). The electrophoresis was run in 1× TAE buffer (40 mM tris buffer (pH 7.6), 20 mM acetic acid, 1 mM EDTA) on a horizontal gel system (BioRad Subcell® GT, USA). This was powered by a BioRad 300 power pack. The electrophoresis was run for 4 h at 40 mA and was then visualised under UV light.

2.15. Single cell gel electrophoresis assay

For the determination of DNA strand breakage, exponentially growing cells were trypsinised and plated at a density of 1×10^6 cells per 24-well plate and allowed to attach for 24 h before incubating with different concentrations of test compound. Concentrations of BNIPSpd were incubated for 4 h and BNIPOSpm were incubated for 4 and 12 h.

After the desired incubation, the medium was aspirated off and cells were rinsed twice with EBSS (Earle's balanced salt solution) before trypsinising. Contents of each well were collected before centrifuging at 2000 rpm for 5 min. Cells were then resuspended in fresh full medium before counting the cells using a haemocytometer. The cells were centrifuged again at 1500 rpm for 3 min. The supernatant was then quickly removed before adding 1 mL MEM supplemented with 20% FCS. Two hundred microlitres of this suspension was then added to 1 mL of cooled PBS and left for 5 min. This

was then centrifuged at 1500 rpm for 3 min. Cells were resuspended in 1% (w/v) LMP agarose before being put on a microscope slide previously coated with 1% (w/v) normal melting point agarose. Microscope slides were placed into cooled slide boxes with lysis solution (pH 10) and left overnight at 4 °C. Slides were then placed in an electrophoresis tank covered with electrophoresis solution (NaOH, EDTA, pH 13, 4 °C) for 40 min to allow DNA unwinding before electrophoresis at 25 V for 30 min at 4 °C. Slides were then placed in neutralising buffer (pH 7.5, 0.4 M tris) at 4 °C for 5 min. This was repeated twice more. Slides were dried at 37 °C before scoring. After drying, 30 μL LMP was added to each gel and allowed to harden before adding 20 μL DAPI (1 μg/mL).

Cells were scored for DNA damage visually. One hundred comets were scored per slide (2 gels per slide) based on the amount of fluorescence in the nucleoid (comet) tail and given a value of 0, 1, 2, 3 or 4 (were undamaged cells are given a score of 0 and maximum damaged cells a score of 4). The total score therefore ranged from 0 to 400. This method of classification has been extensively validated using computerised image analysis. The single cell gel electrophoresis assay was performed three times with four replicates per sample [14].

2.16. Fluorescence microscopy

For the determination of cell morphology after treatment, exponentially growing cells were trypsinised and plated at a density of 3.6×10^4 cells/cm^2 onto sterile coverslips in 60 mm culture dishes. Microscopic analysis was carried out a minimum of three times with each sample having four replicates. Cells were allowed to attach for 24 h before incubating with 1 μM of test compound for different time intervals. After the desired incubation, the medium was aspirated and cultures were rinsed twice with PBS. The coverslips were then mounted onto microscope slides using 90% (v/v) glycerol. To confirm cellular uptake of the compounds, fluorescence microscopy was repeated with the cells at 4 °C rather than 37 °C. Cells were incubated overnight at 37 °C for attachment onto coverslips. They were then incubated at 4 °C for 2 h before the addition of 1 μM of test compound for 6 and 12 h. Coverslips were then mounted onto slides as described above.

2.17. Statistics

A minimum of three independent repeat experiments was conducted for each set of analyses. All experiments comprised three internal replicates. Data is presented as means ± S.D. and where appropriate data was compared by student's *t*-test (Sigma Stat, SPSS software).

3. Results

3.1. Synthesis of bisoxynaphthalimidopolyamines

The synthesis of compounds BNIPOPut, BNIPOSpd and BNIPOSpm is depicted in Fig. 2. The common starting material is *O*-tosylpropyloxynaphthalimide **5** prepared from 3-bromopropan-1-ol **1**. To introduce the aminooxy functionality, 3-bromopropan-1-ol was treated with *N*-hydroxyphthalimide in DMF and in the presence of triethylamine to afford hydroxypropyloxyphthalimide **2**. Removal of the phthalimido-protecting group with hydrazine hydrate at room temperature yielded aminooxypropanol **3**. Treatment of **3** with naphthalic anhydride under basic conditions gave hydroxypropyloxynaphthalimide **4,** which upon reaction with tosyl chloride in anhydrous dichloromethane gave **5**. The mesitylated polyamines **6–8** were synthesized according to our previous report [9,12] and they were treated with **5** in anhydrous DMF and in the presence of cesium carbonate to give the fully protected bisnaphthalimidooxy polyamines. Subsequent removal of the mesityl groups with glacial acetic acid/hydrobromic acid afforded BNIPOPut, BNIPOSpd and BNIPOSpm in 48, 72 and 68% yields, respectively. BNIPOPut, BNIPOSpd and BNIPOSpm all showed good solubility (20% DMSO/water) and we were able to use them in all the biological assays described in this paper.

3.2. Cytotoxic studies

The cytotoxicity of polyamine derivatives was assayed against MCF 7 cells (a breast cancer cell line). Cells (2×10^4) were incubated in the growth media at 37 °C overnight in the presence of the polyamine derivatives at varied concentrations (0.1–50 μM) for 24 and 48 h. The degree of cell viability was analyzed by the MTT assay [15] (Table 1).

When comparing cytotoxity after 24-h drug exposure using the concentration response curves obtained from the MTT assays, similar curves were observed with BNIPSpm and BNIPOSpd. BNIPSpd demonstrated the sharpest decrease in absorbance (see Fig. 3). Increasing compound exposure led to higher cell death except in the case of BNIPOPut. After 48-h drug exposure, BNIPOSpm and BNIPOSpd gave similar IC_{50} of 29.55 ± 8.01 μM and 27.22 ± 6.57 μM, respectively.

It is interesting to note that when BNIPOSpm is exposed to cells for 72 h, an increase in cytotoxicity was observed with an IC_{50} value of 10.0 μM. However both BNIPOSpm and BNIPOSpd were less toxic when compared with BNIPSpm and BNIPSpd with IC_{50} of 5.5 ± 0.5 μM and 0.73 ± 0.17 μM, respectively. The order of toxicity of polyamine derivatives on MCF 7 cells after 48-h drug exposure was BNIPSpd > BNIPSpm > BNIPOSpd > B-BNIPOSpm > BNIPOPut; therefore, the extent of toxicity

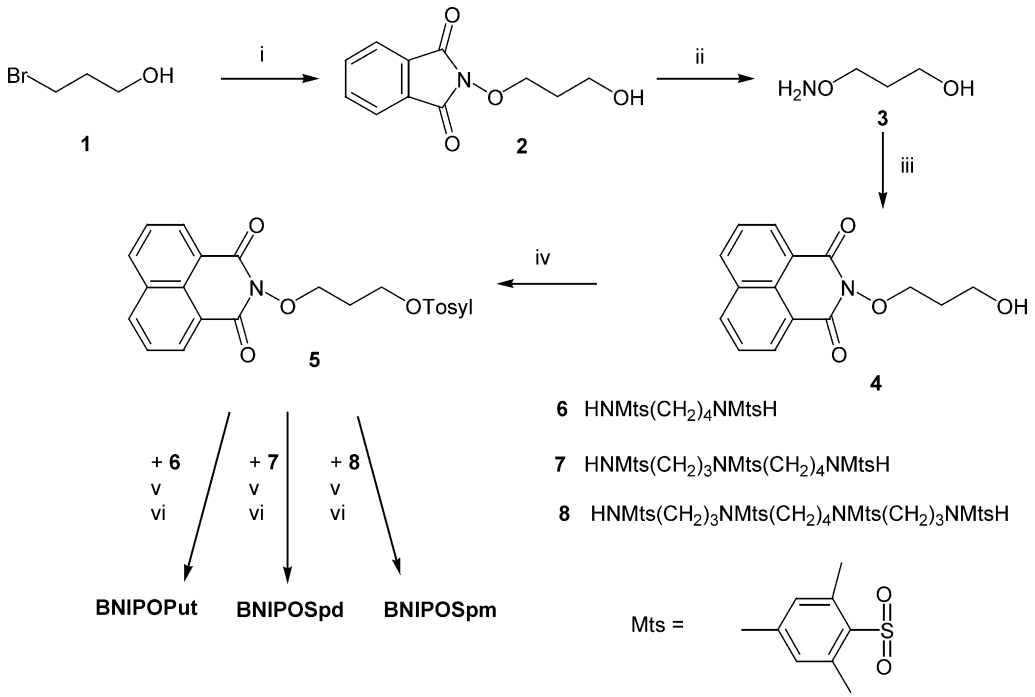

Fig. 2. Reagents and conditions: (i) *N*-hydroxyphthalimide in DMF, Et_3N, 85 °C, overnight; (ii) H_2NNH_2 in abs. ethanol, RT, overnight; (iii) naphthalic anhydride in DMF, DBU, 85 °C, overnight; (iv) tosyl-Cl, CH_2Cl_2, Et_3N, 50 °C, overnight; (v) mesitylated polyamines, DMF, 85 °C, overnight; (vi) HBr/glacial CH_3CO_2H in CH_2Cl_2, RT, 24 h.

depends significantly on the nature of the polyamine chain linking the two naphthalimido rings.

3.3. DNA-binding properties

The determination of melting point, i.e. midpoint of thermal denaturation (T_m) of *calf thymus* DNA duplexes, measured in presence and absence of above compounds and the results are shown in Table 2.

The polyamine derivatives, at a concentration of 10 μM, all increased the T_m of *calf thymus* DNA duplexes indicating a DNA–drug interaction.

The fluorescent ethidium bromide displacement assay (Table 2) was performed to evaluate DNA-binding affinity of compounds BNIPSpd, BNIPSpm, BNIPOSpd, BNIPOSpm and BNIPOPut. Fluorescence of ethidium bromide is markedly enhanced when bound to DNA [16,17]. C_{50} values (drug concentrations necessary to reduce the fluorescence of initially DNA-bound ethidium by 50%) of each compound was measured using 1.66 μM *calf thymus* DNA and 2 μM ethidium bromide at 25 °C. The order of binding strength to DNA is BNIPSpd > BNIPSpm > BNIBNIPOSpd > BNIPOSpm > BNIPOPut (Table 2).

Table 1
Cytotoxicity of polyamines derivatives against MCF 7 cells

Compounds[a]	IC_{50}[b] (μM)	
	24 h	48 h
BNIPSpm	13.32 ± 0.63	5.5 ± 0.5
BNIPSpd	1.50 ± 0.42	0.73 ± 0.17
BNIPOPut	>50	>50
BNIPOSpm	>50	29.55 ± 8.01
BNIPOSpd	32.12 ± 6.14	27.22 ± 6.57

[a] MCF 7 cells were incubated in the presence or absence of the indicated compounds.

[b] IC_{50} is defined as the concentration of compound required to reduce absorbance measured at 560 nm by 50%. Data are means ± S.D. of three replicates.

3.4. Gel mobility assay

Intercalation of a drug between the base pair results in physical unwinding of the DNA which may be detected by retardation of the electrophoretic migration of supercoiled DNA [18] Fig. 4.

All the compounds except BNIPOPut caused DNA unwinding. BNIPOSpd was found to intercalate into DNA more strongly than the other drugs. BNIPSpm, BNIPSpd and BNIPOSpm exhibit no significant difference in their binding strength to DNA, agreeing with the T_m values.

3.5. DNA damage

DNA single strand breakage was measured after exposure to three bisnaphthalimido compounds BNIPSpm, BNIPSpd and BNIPOSpm. This assay is routinely used to study the extent DNA damage in drug treated cells. BNIPSpd and BNIPSpm were chosen since being the most active compounds (IC_{50} = 0.73 ± 0.17 μM and

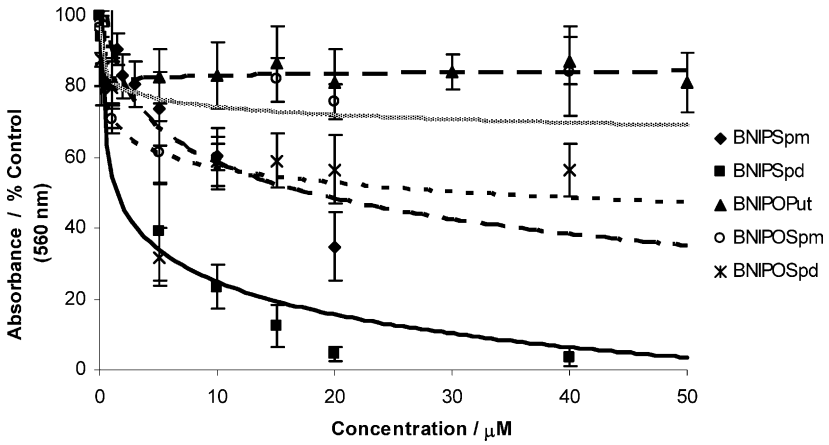

Fig. 3. Dose–response curves. Absorbance determined by the MTT assay and data obtained after treating MCF 7 cells with varying concentrations of compounds (0.01–50 μM) for 24 h. Data are means ± S.D. of six replicates.

5.5 ± 0.5 μM, respectively) and the results obtained, were compared with the moderately active BNIPOSpm (IC_{50} = 29.55 ± 8.01 μM). After 4-h exposure to drug concentrations from 0.01 to 100 μM, BNIPSpm and BNIPSpd were found (Fig. 5) to cause significant DNA damage at 0.1 μM, reaching a maximum effect at 100 μM BNIPOSpm only produced a low, non-dose dependent level of DNA damage from 20 μM (data not shown). However, when BNIPOSpm was exposed to cells for 12 h, significant and extensive dose dependent DNA damage was observed. The extent of DNA damage is similar to BNIPSpm when the latter was exposed to cells for 4 h as illustrated in Fig. 5.

3.6. Intracellular localisation

MCF 7 cells treated with 1 μM of BNIPSpm, BNIPSpd and BNIPOSpm were visualized under a fluorescence microscope after 1, 6 and 24 h. With BNIPSpm and BNIPSpd, some fluorescence can be observed after 2 h while no fluorescence could be detected with BNIPOSpm. After 6 h, the fluorescence with BNIPSpm and BNIPSpd was intense. However with BNIPOSpm, no fluorescence was observed. After 12-h drug exposure, all the three drugs showed intense fluorescence properties as shown in Fig. 6. When the above experiments were performed at 4 °C, no fluorescence was observed in all cases.

Table 2
Effect of drug treatment on the thermal denaturation (T_m) of *calf thymus* DNA and ethidium bromide displacement bound to *calf thymus* DNA (C_{50} values)

Compounds[a]	T_m (°C)[b]	C_{50} (μM)[c]
Calf thymus DNA alone	80.66 ± 0.53	–
BNIPSpm 10 μM	92.21 ± 0.56	3.93 ± 0.12
BNIPSpd 10 μM	95.20 ± 1.59	1.21 ± 0.06
BNIPOPut 10 μM	86.89 ± 2.45	>40
BNIPOSpm 10 μM	93.22 ± 1.84	17.33 ± 3.7
BNIPOSpd 10 μM	97.11 ± 0.39	6.03 ± 0.49

[a] *Calf thymus* DNA was incubated in the presence or absence of compounds for 5 min.
[b] T_m is temperature (°C) when half of the DNA is denaturated.
[c] C_{50} is drug concentrations required to reduce the fluorescence of initially DNA-bound ethidium by 50%. Data are means ± S.D. of three replicates.

4. Discussion

Three bisoxynaphthalimido polyamine derivatives (BNIPOPut, BNIPOSpd and BNIPOSpm) were synthesized and were found to have good solubility properties. It would appear that the presence of several heteroatoms in the linker chain enhances the solubility of bisnaphthalimido compounds. However, the presence of oxygen atoms in the linker chains tends to cause a decrease in cytotoxic activity.

It is interesting to note that BNIPOSpm and BNIPOSpd intercalate as effectively to calf thymus DNA as BNIPSpd

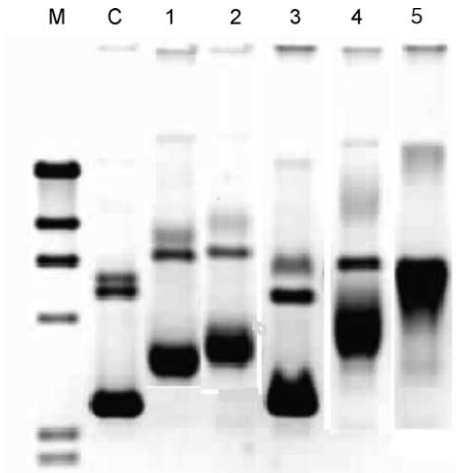

Fig. 4. Effect of oxa-polyamines on the mobility of the supercoiled pBR 322. All lanes contained 1 μg of pBR 322. Lane M, molecular marker; lane C, pBR 322 only; lane 1, BNIPSpm (10 μM); lane 2, BNIPSpd (10 μM); lane 3, BNIPOPut (10 μM); lane 4, BNIPOSpm (10 μM); lane 5, BNIPOSpd (10 μM).

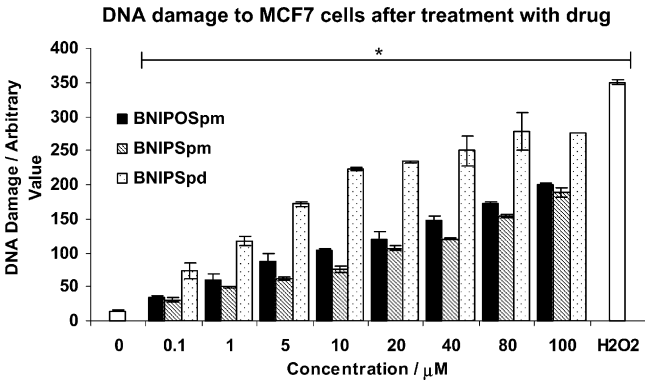

Fig. 5. DNA damage (strand breakage) determined by Single Cell Gel Electrophoresis. Data obtained after treating MCF 7 cells with BNIPSpm, BNIPSpd for 4 h and BNIPOSpm for 12 h at 5 μM drug concentration. H_2O_2 (200 μM) was used as a positive control. Data are means ± S.D. of three replicates, $^{*}P < 0.001$ vs. negative control.

as demonstrated by the T_m value and the gel mobility assay. However, BNIPSpd being the most active compound showed the highest efficiency in displacing intercalating agent, ethidium bromide from DNA and also caused maximum DNA damage at low drug concentration. The low toxicity of BNIPOSpm and BNIPOSpd may be explained by their less effective uptake compared to BNIPSpm and BNIPSpd. This is supported that by the microscopic observations of no fluorescence in BNIPOSpm treated cells for 6 h (Fig. 6). In contrast, BNIPSpm and BNIPSpd showed high fluorescence and toxicity.

It is important to note that the cytoxicities of BNIPSpm, BNIPSpd and BNIPOSpm do not correlate directly with the data obtained on DNA damage. For example after 12-h drug exposure, BNIPOSpm inflicted more DNA damage when compared with BNIPSpd and yet the IC_{50} value of BNIPOSpm is >50 μM after 24-h drug exposure. This may suggest the participation of many other intricate factors that are involved in inducing DNA damage and cytotoxicity.

The subtle difference between the structures of BNIPSpm and BNIPOSpm (Fig. 1) revealed large differences in their cytotoxicity against MCF 7 cells. Because all the compounds discussed in this paper are polycations, we reason that like other polyamine analogues [19,20], these compounds could be transported through the cell by an active transport system, e.g. the polyamine transporter. It is worth mentioning that little is known about the characterization of the polyamine transporter [21]. This theory is further supported by the apparent absence of drug uptake as indicated by the lack of fluorescence at low temperature of 4 °C (data not shown).

We conclude that the introduction of oxygen atom in the α-position of the naphthalimido ring like in the case of BNIPOPut, BNIPOSpd and BNIPOSpm, may have resulted in the compounds not being recognized by the polyamine transporter. Therefore the bisoxynaphthalimidopolyamines BNIPOPut, BNIPOSpd and BNIPOSpm entered the cells perhaps by some other routes, e.g. very slow passive diffusion resulting in their delayed and reduced cytotoxicities. We are currently investigating the mechanism of how these compounds are being transported in cancer cells. Furthermore, while the ability of these compounds to bind to DNA may play a role in their activity, there are also other factors contributing to their biological properties.

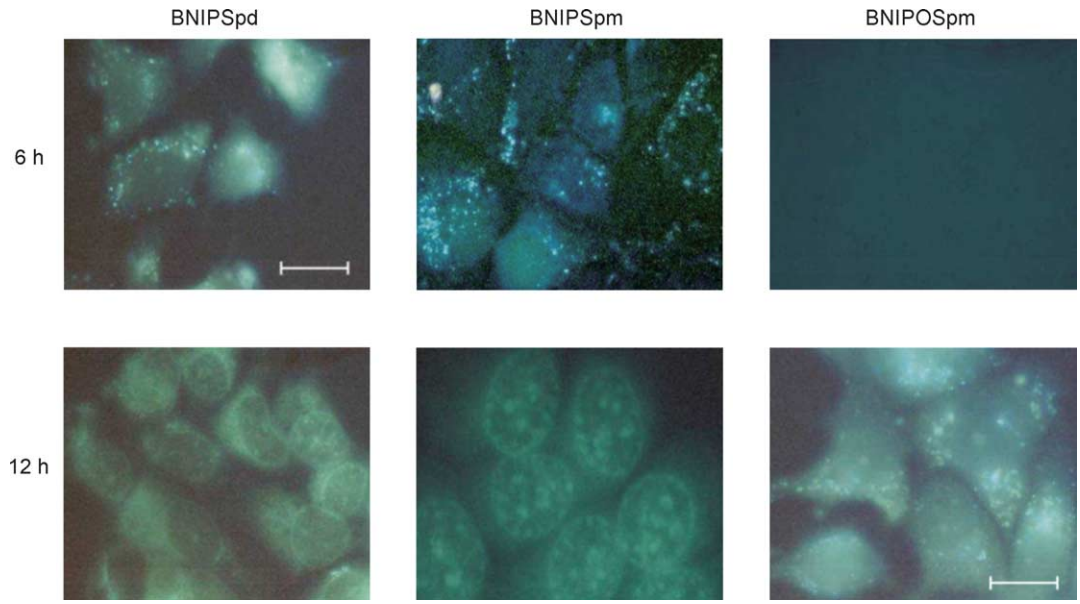

Fig. 6. Fluorescence microscopy images of MCF 7 cells treated with 1 μM BNIPSpd, BNIPSpm and BNIPOSpm for 6 and 12 h. Cells (1×10^6) were grown on sterile coverslips (22 mm × 22 mm) and allowed to adhere for 48 h. Medium was removed and fresh medium added with the drug to a final concentration of 1 μM and incubated for the desired time point then washed twice with PBS and put on slide using 90% glycerol as mountant. Images are representative of eight repeat experiments each comprising three internal replicates.

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