Caspase-independence and characterization of bisnaphthalimidopropyl spermidine induced cytotoxicity in HL60 cells.

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Abbreviations: BNIPSpd, Bisnaphthalimidopropylspermidine; PI, Propidium iodide; OPA, o-phthaldialdehyde.

Running title: Bisnaphthalimide-induced cytotoxicity

Abstract -

Bisnaphthalimides are DNA intercalators of potential use as chemotherpeutics but for which the range of mechanism of action is only gradually being elucidated. Using human promyelocytic HL-60 cells, we extend characterisation of the cytotoxicity of bisnaphthalimidopropylspermidine (BNIPSpd) and examine the relationship with caspase-activity. Within 4h exposure, BNIPSpd (1-10 μM) induced significant DNA strand breakage. Evidence of apoptosis was progressive through the experimental period. Within 6h, BNIPSpd increased the proportion of cells exhibiting plasma membrane phosphatidylserine exposure. Within 12h, active caspase expression increased and was sustained with 5 and 10 μM BNIPSpd. Flow cytometric analysis revealed caspase activity in cells with and without damaged membranes. By 24 h, 5 and 10 μM BNIPSpd increased hypodiploid DNA content and internucleosomal DNA fragmentation (DNA ladders) typical of the later stages of apoptosis. 1μM BNIPSpd exposure also increased hypodiploid DNA content by 48h. Polyamine levels decreased by 24 h BNIPSpd exposure. The pan-caspase inhibitor, z-VAD, significantly decreased DNA degradation (hypodiploid DNA and DNA ladders) and induced an increase in cell growth. Despite this, cell growth and viability remained significantly impaired. We propose that BNIPSpd cytotoxicity arises through DNA damage and not polyamine depletion and that cytotoxicity is dominated by but not dependent upon caspase driven apoptosis.

Key Words: Apoptosis; Bisnaphthalimides; Caspase-inhibition; Cytotoxicity; Genotoxicity; HL-60 cells

1. Introduction

Naphthalimides and bisnaphthalimides are DNA intercalating agents (e.g. Brana et al., 1993, 2001; Cacho et al., 2003; Kong Thoo Lin et al., 2003; Lv et al., 2009; Tan et al., 2015; Wang et al., 2016) with compounds comprising these moieties variously being proposed for exploitation as anti-tumourigenics, anti-microbial or anti-parasitic agents (e.g. Gellerman, 2016; Kong Thoo Lin et al., 2003; Oliveira et al., 2007; Noro et al., 2015; Kopsida et al., 2016 Graca et al., 2016). Bisnaphthalimides consist of two aromatic naphthalimido rings attached by a linker chain containing nitrogen atoms. Principally, the intercalations of the naphthalimide planar aromatic rings between DNA base-pairs distorts the conformation of the DNA backbone leading to interference with DNA-protein interactions (Hsiang et al., 1989; Brana et al., 1993, Dance et al., 2005). In addition, particular naphthalimide and bisnaphthalimides have been variously shown to inhibit toposiomerases directly, exert post- DNA damage effects on DNA damage signalling pathways, impair DNA repair, or induce lysosomal permeability (Filosa et al., 2009; Zhu et al., 2009; Chen et al., 2010; Bestwick et al., 2011; Barron et al., 2015; Tan et al., 2015; Zhang et al., 2016a;). The ultimate consequence of exposure to (bis-) naphthalimides *in vitro* has included cell cycle arrest and apoptosis (Ralton et al., 2009; Liang et al., 2011; Yang et al., 2011a; Seliga et al., 2013; Zhang et al., 2016a).

This array of often complementary mechanisms of (bis-)naphthalimide action influencing tumour cell, microbes and parasite development has continued to encourage their development as therapeutics. In previous work, we linked bisnaphthalimido propyl fragments with natural polyamines such as spermine and spermidine (Dance et al., 2005; Kong Thoo Lin et al., 2000; Pavlov et al., 2001). As neoplastic transformation can be accompanied by elevated polyamine levels resulting from altered biosynthesis, catabolism and uptake (Basuroy and Gerner 2006), we hypothesised that the polyamine linker would facilitate uptake of the bisnaphthalimides into neoplastic cells (Dance et al., 2005).

We previously reported that bisnaphthalimidopropylspermine (BNIPSpm) and bisnaphthalimidopropylspermidine (BNIPSpd) have enhanced aqueous solubility, and are rapidly and homogeneously distributed within the nuclei of MCF-7 breast carcinoma and Caco-2 colon adenocarcinoma cells where they cause significant DNA damage and impairment of DNA base excision repair (Bestwick et al., 2011; Dance et al., 2005). Moreover, the more cytotoxic of the two, BNIPSpd (Fig 1), induces apoptosis in Caco-2 and HT-29 colon epithelial cells (Ralton et al., 2009). However, the conservation of the apoptotic response to BNIPSpd, and the mechanism underlying such response in differing cell types, has not been established. Here, following a preliminary report (Kong Thoo Lin et al., 2003), we provide a detailed assessment of the effects of BNIPSpd on cell growth and cytotoxicity in HL-60 promyelocytic leukaemia cells, examining the temporal relationship of apoptosis to DNA damage and polyamine levels and the extent of caspase dependency within overall BNIPSpd toxicity.

2. Materials and methods

2.1. Materials

HL-60 cells were purchased from the European Collection of Cell Cultures (98070106; Public Health England, Salisbury, UK). RPMI medium and foetal calf serum were from Lonza Sales AG (Verviers, Belgium). Tissue culture flasks were supplied by Greiner Bio-One Ltd (Gloucesterhire, UK). Active Caspase-3 detection kit, Cell Cycle Plus DNA Reagent Kit and QC DNA particles were from BD (Oxford, UK). Vybrant FAM Polycaspases kits, Amplex Red hydrogen peroxide assay kits and DAPI were from Life Technologies Ltd (Paisley, UK). The Annexin V-FITC kit and single strand DNA detection kit were from eBioscience Ltd (Hatfield, UK). Etoposide, camptothecin, dimethylsulfoxide

(DMSO) and all other reagents were purchased from Sigma-Aldrich Company Ltd (Dorset, UK) unless stated otherwise. BNIPSpd was synthesised and characterised according to our previous methods (Kong Thoo Lin and Pavlov 2000).

2.2. Cell Culture and bisnapthalimidopropyl polyamine exposure

HL 60 cells were cultured in RPMI 1640 medium supplemented with 10 % (v/v) foetal calf serum, 1% (v/v) non essential amino acids, 2 mM glutamine, 50 μg/mL streptomycin and 50 μg/mL penicillin. Cells were kept in a humidified (95% relative humidity, RH) incubator at 37 °C, 5% CO_2. BNIPSpd was solubilized in 20% (v/v) DMSO and cells ($5x10^5$ cells mL^{-1}) were incubated with 0.1-10μM BNIPSpd (0.02% [v/v] DMSO final concentrations), 0.02 % (v/v) DMSO or sterile double distilled water for 1-72 h. The chemotherapeutic agents etoposide (10 μM) and camptothecin (4 μM) in 0.1% [v/v] DMSO final concentrations, were used as positive controls to confirm assay function as appropriate. For caspase inhibitor experiments, cells were pre-incubated with 100 μM z-VAD-fmk (in 0.2% v/v DMSO final concentration) for 1 h at 37°C, 5% CO_2, 95% RH prior to addition of BNIPSpd.

2.3. Cell culture growth and cytotoxicity

Cells were collected by centrifugation (300 g for 5 min, RT), the culture media removed and the pellet suspended in trypan blue (0.2% w/v final concentration in PBS). Cells were counted using a Neubauer haemocytometer (Baur et al., 1975).

2.4. DNA single strand breaks

DNA single strand breaks were determined by single cell gel electrophoresis (SCGE) as described previously (Bestwick et al., 2005). Nucleoids were stained with 4',6-diamidino-

2-phenylindole (DAPI, 5 μgmL^{-1} stock) and scored visually. One hundred images per gel, (with duplicate gels per slide) were classified according to the intensity of fluorescence in the nucleoid tail and assigned a value of 0-4 with 0 representing no damage and 4 maximal damage. Thus, the total damage score can range from 0 to 400. This method of classification has been extensively validated using computerised image analysis (Duthie et al., 1996).

2.5. Phosphatidylserine exposure and membrane integrity

Exposure of phosphatidylserine at the extracellular surface of the plasma membrane was determined as previously described (Bestwick and Milne 2006) by FITC-Annexin-V binding using a commercial assay kit, and the relationship to membrane damage assessed by co-incubation with propidium iodide (PI) as per the manufacturer's protocol (eBioscience Ltd, Hatfield, UK).

2.6. Caspase-3 and pan-caspase

The presence of the active fragment of caspase-3 was analysed by flow cytometry after cell fixation, permeabilization and labelling with PE-conjugated polyclonal rabbit anti-active caspase-3 (BD, Oxford) as described previously for HL-60 cells (Bestwick and Milne 2006). Total (pan-) caspase activity relative to maintenance of membrane-integrity was estimated by measuring fluorescence in cells co-treated with FAM-VAD-FMK polycaspase reagent (labelling active caspase 1, 3, 4, 5, 6,7, 8, 9) and propidium iodide according to manufacturer's recommendations (Life Technologies Ltd, Paisley, UK). Camptothecin treatment and mock-(water) treated cells (positive and negative controls respectively), were used to define active caspase-3 expression or increased pan-caspase activity using Cell Quest software (BD, Oxford, UK). 10,000 events were recorded.

2.7. Apoptosis-associated DNA fragmentation

SubG1 (hypodiploid) DNA content was evaluated as described previously (Bestwick et al., 2007) using a commercial kit (Cycle test plus) according to the manufacturer's protocol (BD, Oxford, UK). Flow cytometry of the propidium iodide-stained nuclei was performed with a flow rate of 12 µL/min and cell cycle distribution selected from linear FL-2 area v. width plots with doublet discrimination in FL-2. Singlet events, excluding debris, were gated and 20,000 events were acquired within the gate. The percentage of cells with DNA content <2N (sub-G1) was calculated from histograms of linear FL-2 area plots of the singlet gated region using Cell Quest Software (BD, Oxford, UK) and Mod Fit LT software (Verity Software House, ME, USA). A DNA QC Particle Kit (BD, Oxford, UK) was used to verify instrument linearity, doublet discrimination and cytometer alignment.

Internucleosomal DNA fragmentation giving rise to the characteristic apoptotic 'DNA ladder', was identified following DNA extraction and separation by agarose gel electrophoresis as detailed in Bestwick and Milne, (Bestwick and Milne 2006).

2.8 Polyamine extraction and analysis

Cells were treated either with solvent vehicle (DMSO), BNIPSpd or α-difluoromethylornithie (DFMO; 5 mM) as a positive control for polyamine changes. Culture medium from control and BNIPSpd-treated cells were decanted, the cells collected by centrifugation (1000 g, 5 min, 20 ^{o}C) and counted using a Neubauer haemocytometer. Cells were washed with PBS (x2) before being homogenised with perchloric acid (final concentration 1% v/v) and incubated with internal standard (1,7-diaminoheptane ;20 µM for 60 min on ice). The homogenate was centrifuged at 10000g (4 ^{o}C) for 10 minutes and the supernatant transferred to a fresh container and stored at -20^{o}C. The residual cell pellet and culture medium were also retained at -20^{o}C. HPLC analysis of polyamine content was carried out by derivatization with o-phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol as described by Seiler and Knodgen (Seiler and Knodgen, 1985). HPLC was conducted with a Phenomenex Luna 5 mm, 25 cm x 4.6 mm, C18 HPLC column. Detection of polyamines after OPA derivatisation was at 345 nm excitation with 455 nm emission filters. Data were collected and integrated using Waters Empower Software (Waters Yvelines Cedex, France).

2.9. *Hydrogen peroxide determination.*

Hydrogen peroxide, arising from the interaction of the polyamine analogue with amine oxidases in bovine serum (Parchment et al., 1990), is a potential confounder of the cellular response to BNIPSpd. Using the Amplex Red Assay (Life Technologies Ltd, Paisley, UK), the level of hydrogen peroxide in complete RPMI 1640 medium containing BNIPSpd (1-50 μM) was measured within 5h incubation via the peroxidase catalysed formation of resorufin (560 nm,Unicam UV/Vis spectrophotometer) . Co-incubations with native or heat denatured (5 min, 100 $^{\circ}$C) bovine catalase (Sigma-Aldrich Company Ltd, Dorset, UK; 2.5 μg/mL) were used to confirm detection of hydrogen peroxide.

2.10 Statistical Analysis

Experiments were conducted a minimum of three times unless stated otherwise. Data are presented as mean ± SD. A *P*-value of < 0.05 (by student's t-test) was taken as the minimum basis for assigning significance. Statistical analysis was conducted using SigmaStat (Systat, Software Inc. London, UK).

3. Results

3.1. Cell growth and cytotoxicity

BNIPSpd (1-10 μM) caused a significant inhibition (1 μM) or cessation (5 and 10 μM) of cell growth (Fig 2A). Based on trypan blue staining, 5 and 10 μM BNIPSpd also exerted significant cytotoxicity (Fig 2B) within 24 h. Therefore, analysis of the mechanisms underpinning cytotoxicity was predominantly conducted within the first 24 h of treatment.

3.2. DNA single strand breaks

DNA single strand breakage was significantly increased after 4h exposure to 1 μM-10 μM BNIPSpd and remained significantly increased over control cells after 24h treatment (Fig. 3).

3.3. Phosphatidylserine exposure and membrane integrity.

Exposure to BNIPSpd (≥ 1 μM) caused a significant increase in the proportion of cells binding annexin-V-FITC at 6-24 h but remaining recalcitrant to PI staining (Fig 4A and 4B). This indicates exposure of phosphatidylserine on the external plasma membrane surface in the absence of membrane damage. In addition, BNIPSpd also induced a significant increase in the proportion of cells co-labelling with annexin-V-FITC and PI (Fig.4A and B), representing annexin-V-FITC binding to cells with damaged membranes. Cells treated with solvent vehicle alone remained predominantly unlabeled with either annexin-V-FITC or PI (Fig 4A and B). For all treatments, a residual proportion of cells stained with PI but did not label with annexin-V-FITC. This represented 2.6 % (± 0.19) of control cells at 24 h and this increased ($P < 0.05$) after treatment with 10 μM BNIPSpd (24 h) to14.3 % (± 3.4).

3.4. Active caspase-3 expression and pan caspase activity.

Active caspase-3 expression increased during time in culture within negative (DMSO solvent only treated) control cells but 10μM and 5 μM BNIPSpd treatment further significantly enhanced and sustained active caspase-3 expression by 4 h and 12h after exposure respectively (fig 5). A transient but significant increase in caspase-3 was also observed by 12 h of incubation in 1μM BNIPSpd treated cells (Fig. 5).

Focussing on 5 and 10 μM BNIPSpd treatment for which there was a more rapid and sustained active caspase-3 expression (Fig 5), the relationship between general-caspase activity and membrane damage was flow-cytometrically assessed by combining FAM-VAD-FMK FLICA labelling of active caspases (caspase 1, 3, 4, 5, 6,7, 8, 9) with PI staining to measure membrane integrity. The median fluorescence intensity, as a marker of overall caspase activity (Fig 6A) and the proportion of cells expressing pancaspase activity (Fig 6B) increased significantly by 8 h of treatment with BNIPSpd. Increased pan-caspase activity was detected both in cells with intact membranes and in those with compromised membrane integrity (Fig 6B).

3.5 'Apoptotic' DNA fragmentation

A significant increase in the proportion of cells with a subG1 DNA content was observed after 3 h and 24 h for 10 and 5 μM BNIPSpd treatments respectively. A smaller but nevertheless significant increase in sub G1 DNA content was observed by 48 h with 1 μM BNIPSpd (Fig. 7A). Classical apoptotic internucleosomal DNA fragmentation was not observed within the first 12 h of BNIPSpd exposure. By 24 h, however, DNA ladders occurred in cultures treated with ≥ 5 μM BNIPSpd (Fig. 7B) and were particularly apparent in 5 μM BNIPSpd treated cells

3.6. Polyamine levels

No changes to spermine and spermidine levels were observed after 12 h incubation with 1-10 μM BNIPSpd. BNIPSpd treatment for 24 h resulted in a lowering of spermine and spermidine levels (Fig 8A and B). Putrescine was not detected in the analysis of either BNIPSpd treated or untreated cells.

3.7. Effect of caspase inhibitor on cytotoxicity

To investigate caspase-dependency of BNIPSpd toxicity, cells were pre-treated with the pan caspase inhibitor z-VAD-FMK, prior to and during exposure to 10 μM BNIPSpd. Z-VAD-fmk strongly decreased the intensity of internucleosomal DNA fragmentation and significantly lowered the increase in sub G1 DNA content (Fig 9 A and B). The anti-proliferative (Fig 9C) and cytotoxic effects (Fig 9D) of BNIPSpd were slightly but significantly lowered by caspase inhibition. Nevertheless, there remained a highly significant reduction in cell growth (Fig 9C) and high levels of cytotoxicity (Fig 9D) in response BNIPSpd.

3.8. Culture medium hydrogen peroxide content

The influence of BNIPSpd on the concentration of hydrogen peroxide in serum-supplemented culture media, which may be a confounding factor in BNIPSpd toxicity, was established via the Amplex Red assay. Endogenous hydrogen peroxide present in the test mixture acts as a co-substrate in the oxidation of amplex red via exogenously added horseradish peroxidase and leads to the formation of resorufin. No significant change in hydrogen peroxide content was observed up to 10 μM BNIPSpd (the maximum concentration used in the cytotoxicity assays) but a significant increase in resorufin absorbance was observed with 50 μM BNIPSpd. This increase was sensitive to the presence of native catalase, confirming the potential for elevated levels of exogenous hydrogen peroxide at these higher, but here, not experimentally relevant BNIPSpd incubations (Fig 10).

4. Discussion

Within a series of novel bisnaphthalimido-polyamine compounds, the spermidine-containing BNIPSpd exerts the dominant cytotoxicity against MCF-7 breast carcinoma (Dance et al., 2005) and Caco-2 colon adenocarcinoma cells (Bestwick et al., 2011). Cytotoxicity is preceded by DNA single strand breakage (Dance et al., 2005; Bestwick et al., 2011). Here, BNIPSpd also rapidly caused DNA single strand breaks in HL-60 cells and a progressive increase in features typical of a classical apoptotic progression towards cell death. Within 24h, BNIPSpd exposure was associated with early externalisation of plasma membrane phosphatidylserine, a progressive increase in active caspase-3 and pan caspase activity and DNA fragmentation (hypodiploid DNA content and internucleosomal DNA fragmentation), all features indicative of apoptosis (Elmore 2007; Darzynkiewicz and Li 1996; Kohler et al., 2002; Otsuki et al., 2003).

Both the naphthalimide and polyamine moiety of BNIPSpd may trigger apoptosis (Basuroy and Gerner 2006; Casero and Woster, 2001; Brana et al., 2001; Liang et al., 2011). For the (bis)naphthalimide moiety, DNA intercalation and the effect on DNA replication and integrity is proposed as the primary mode of action (Kong Thoo Lin and Pavlov, 2000; Dance et al., 2005) but the presence of the polyamine moiety might also suggest an influence on cellular polyamine levels and metabolism (Morgan, 1999; Wallace and Niiranen, 2007; Schipper et al., 2000; Basuroy et al., 2006). The naturally occurring polyamines putrescine, spermidine and spermine represent a potential link between proliferation and cell death (Schipper et al., 2000; Moschou et al., 2014) and polyamine analogues may influence cell survival by effects on cellular polyamine pools (Zou et al., 2004). However, polyamines exhibit a complex relationship to cell survival and exert both negative and positive regulatory effects on apoptosis (e.g. Milovic and Turchanowa, 2003; Stefanelli et al., 2001; Yuan et al., 2002; Zou et al., 2004) .

Here, DNA single strand breakage occurred within the first 4 h of treatment with BNIPSpd. This parallels or occurs in advance of the earliest detection of apoptosis markers. Conversely, there was no change in polyamine level within the first 12 h of exposure. Thus, while polyamine levels do decline during cell death, on a temporal basis polyamines do not appear to be involved in induction of DNA damage or initiation of apoptosis within BNIPSpd-treated HL60 cells. This contrasts with observations in Caco-2 and HT-29 cells where polyamine levels are significantly affected both at the pro-apoptotic BNIPSpd dosage range (>0.5 μM) and by non- toxic BNIPSpd exposures ($\geq$ 0.01 μM) (Ralton et al., 2009). Based on our observations of the timing of BNIPSpd- induced DNA damage relative to changes in polyamine levels, we propose that it is the effect of the bisnaphthalimide moiety on DNA (Dance et al., 2005; Bestwick et al., 2011) that is the consistent early-induced phenomenon associated with BNIPSpd-cell interactions.

However, when considering the extent of cytotoxicity and DNA strand breakage at 1 μM BNIPSpd exposure, the degree of DNA damage does not predict the eventual magnitude of apoptosis or overall toxicity, with limited toxicity despite considerable DNA damage and inhibition of proliferation. Inhibitory effects on DNA repair activity may predominate at these BNIPSpd concentrations (Bestwick et al., 2011). The influence of BNIPSpd and other analogues on cell growth and survival regulation at low-cytotoxic and sub-cytotoxic dosages are currently under investigation.

In addition to the direct effect of BNIPSpd on cell survival, we considered whether amine oxidases in the culture medium may promote hydrogen peroxide generation in the presence of the spermidine analogue (Bitonti et al., 1990; Parchment et al., 1990). No increase in hydrogen peroxide was detected in culture medium incubated with BNIPSpd at the concentrations employed. Consequently, hydrogen peroxide generation from BNIPSpd addition in the media appears not to be a factor in BNIPSpd toxicity in this culture system. However, whether BNIPSpd toxicity involves oxidative stress intracellularly has not been determined.

Given the timing of DNA strand break detection, we suggest that the intrinsic mitochondrial pathway for apoptosis, initiated by DNA damage as a likely primary route for orchestrating toxicity. Given the p53 null status of HL60 (Leroy et al., 2014), this is not dependent on p53 though be mediated through a functional substitution such as via p73 (e.g. Chakraborty et al., 2010), a member of the p53 protein family (Chekova et al., 2018). Although BNIPSpd toxicity was associated with features typical of a classical apoptosis, the presence of a proportion of cells exhibiting membrane damage concomitant with or in advance of apoptotic markers also suggests a more heterogeneous cell death process occurs as part of the overall BNIPSpd-induced toxicity. Cytotoxicity of BNIPSpd in MCF-7 cells (Dance et al., 2005) which do not express Caspase-3 (Janicke, 2009) indicates a non-caspase-3 dependency for toxicity. Here, z-VAD-FMK was used to investigate general caspase-relationship to cytotoxicity. While abolishing apoptotic DNA fragmentation, z-VAD-FMK failed to rescue the majority of cells from the anti-proliferative and cytotoxic effects of BNIPSpd. Many cytotoxic chemotherapeutic drugs demonstrate non-caspase dependency (e.g. camptothecin, doxorubicin, paclitaxel; Broker et al., 2005). Caspase inhibition can induce a switch from classical apoptosis to alternate cell death processes or an alteration of dominance of a cell death mechanism paralleling apoptosis induction. The range of changes to cell death mode has included autophagy, “mitotic catastrophy”, necrosis-like programmed cell death (e.g. necroptosis; Zhang et al., 2016b) and more traditional necrosis (Broker et al., 2005). For example, caspase inhibition switches doxorubicin-induced cytotoxicity within SKN-SH neuroblastoma cells from apoptosis to senescence (Rebbaa et al., 2003), whereas Z-VAD-FMK promotes a shift from apoptosis to necrosis during corneal scrape-induced keratocyte death (Kim et al., 2000). For many of these outcomes, the mechanistic inter-relationship has yet to be comprehensively established. Caspase inhibition might also increase the role of non-caspase dependent apoptotic-like processes, for example involving apoptosis inducing factor (AIF). AIF has been implicated recently in mono-naphthalimide spermidine-induced apoptosis in HeLa cells (Yang et al., 2011b). We hypothesise that the many influences of (bis)naphthalimide-containing compounds on processes linked to cell proliferation and genomic integrity (Filosa et al., 2009; Zhu et al., 2009; Chen et al., 2010; Bestwick et al., 2011; Yang et al., 2011a; Li et al., 2013; Seliga et al., 2013; Shen et al., 2013) renders them capable of inducing a range of cell death mechanisms as well as multiple point of provocation of apoptosis.

Here HL-60 cells are utilised but comparison to multiple cell types (and ultimately the use of organoid systems) will help to confirm the universality and mechanistic variations of BNIPSpd-cell-interactions in vitro. Nevertheless, the effects observed in Caco-2 and HT-29 colon adenocarcinoma cell cultures (Ralton et al. 2009) point to key conserved outcomes in terms of apoptosis features and early induction of DNA damage post exposure (Bestwick et al., 2011) but, as discussed above, significant differences are apparent for the effect on cellular polyamine content (Ralton et al., 2009). Significantly, previous work on characterising BNIPSpd and related bisnaphthalimide-polyamine compounds (Dance et al., 2005) on the form of toxicity within caspase-3 deficient but p53 active MCF-7 cells (e.g. Zhao et al., 2017) would be a useful component of comparisons on BNIPSpd activity within differing apoptotic regulator-competent backgrounds.

In conclusion, BNIPSpd cytotoxicity in HL60 cells occurs primarily through an apoptotic-like process, potentially initiated by bisnaphthalimide-induced DNA damage and within a temporal framework involving phosphatidylserine exposure, caspase activation and DNA fragmentation. However, caspase inhibition does not rescue cells from BNIPSpd toxicity. While it remains to be established whether this adds to exploitability for chemotherapy or represents a confounder to the therapeutic development of this bisnaphthalimidopropyl polyamine series, caspase-independent cell death provides further evidence of the heterogeneity in the cytotoxic response to (bis)naphtahlimides.

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Figure Legends

Fig 1. Structure of Bisnaphthalimidopropylspermidine (BNIPSpd)

Fig 2. Cell growth (A) and cell death (B) were determined following exposure to DMSO (□), 1 μM (○), 5 μM (⊕) and 10 μM (●) BNIPSpd. Cell counts were determined by microscopy, with trypan blue staining used to indicate loss of membrane integrity. Data are means ± SD (n ≥6). *P<0.05, **P < 0.01, ***P < 0.001 v. respective time point for DMSO control.

Fig. 3. DNA damage. Alkaline SCGE followed by visual ‘comet’ scoring was used to determine DNA single strand breaks after exposure to BNIPSpd. Data are means ± SD (n=3), ***P<0.001 v. respective time point for DMSO control.

Fig 4. Effect of BNIPSpd exposure on plasma membrane phosphatidylserine labelling relative to membrane damage. Cells were treated with BNIPSpd for 6 (A) and 24h (B). Cells labelled with Annexin-V-FITC but resistant to PI uptake (Annexin-V+) represent those with plasma membrane extracellular surface exposed phosphatidylserine without plasma membrane damage and those stained with both Annexin-V-FITC and PI (Annexin-V+/ PI+) also have damaged plasma membranes. Data was obtained by flow cytometry and 10,000 events were analysed per sample. Data are means ± SD (n=3) * P<0.05, ** P = 0.002,***P<0.001 for increases v. DMSO control.

Fig 5. Detection of the ‘active’ form of caspase-3. The level of active caspase-3 expression was immuno-flow cytometrically determined 4, 12 and 24h after treatment with DMSO and 1, 5 and 10 μM BNIPSpd. 10,000 events were analysed per sample. Data are means ±SD (n=6). * P=0.05 or *** P<0.001 for comparison v. the respective DMSO control.

Fig 6. The relationship between general (pan)-caspase activity and membrane damage. FAM-VAD-FMK FLICA labelling with PI co-staining was used to flow cytometrically assess the level of caspase activity relative to membrane damage within 8h of treatment with BNIPSpd. The median fluorescence intensity from FAM-VAD-FMK FLICA labelling was used as a marker of cellular pan-caspase activity (A). The proportion of cells with FAM-VAD-FMK FLICA but no PI fluorescence (C+), co-fluorescing with FAM-VAD-FMK FLICA and PI (C+, PI+) , fluorescing only due to PI (PI+) or showing no fluorescence with FAM-VAD-FMK FLICA and PI (NR) was used to establish the relationship between caspase activity and the occurrence of membrane damage (B) the latter as indicated by heightened PI fluorescence. 10,000 events were analysed per sample. Data are means ±SD (n=3). * P<0.05 or *** P<0.001 for comparison v. the staining within the respective DMSO control.

Fig 7. ‘Apoptotic’ DNA fragmentation. Sub G1 (hypodiploid) DNA content (A), as an indicator of apoptotic DNA fragmentation, was determined from flow cytometric analysis of PI staining using ModFit LT software (Verity Software House, ME, USA). Cells were treated with DMSO or BNIPSpd. Data are means ± SD (n=3). * P<0.05, **P<0.01, *** P<0.001 for comparison v. the respective DMSO control. Internucleosomal DNA fragmentation (B), considered a classical feature of apoptosis, was detected by agarose gel electrophoresis following 24h exposure to DMSO, 10μM etoposide (positive control for ladder formation) or BNIPSpd (1, 5 or 10μM). M, markers; C no treatment, D, Solvent (DMSO); E, etoposide.

Data is a representative image from one of three experiments (note image split for presentation).

Fig 8. Influence on cellular polyamine levels. Effects of polyamine-analogue exposure on spermine and spermidine was evaluated by HPLC after 12 (A) and 24 (B) h exposure to BNIPSpd. As an internal control, effects of polyamine analogues are compared with those of α-difluoromethylornithine, DMFO (5mM), an ornithine decarboxylase inhibitor. SPD, spermidine; SPM, spermine. Data are means ± SD (n=3). *P<0.05 or **P<0.01 v. DMSO control.

Fig 9. The influence of caspase inhibitor z-VAD-fmk on DNA fragmentation and cell death. Cells were pre-treated with the cell permeable pan caspase inhibitor, z-VAD-FMK, prior to and during exposure to 10 μM BNIPSpd. The extent of DNA ladders (A): lane M, Mw markers; lane 1, no solvent vehicle or inhibitor; lane 2, DMSO (solvent vehicle) treatment only; lane 3, z-VAD-FMK; lane 4, BNIPSpd; lane 5, BNIPSpd and z-VAD-FMK lane 6, etoposide (10μM) as positive control for DNA ladder (apoptosis) formation. Note gel-image splicing is for presentation; (B): the proportion of cells with hypodiploid DNA content, (C): the effect on cell proliferation and (D): cell death as determined at 24h from start of exposure. Data is either a representative image from three experiments (A), or are means ± SD (n=3), data designated by a different letter is significantly different (P<0.05) relative to control or treatment at the respective time point.

Fig 10. BNIPSpd induced exogenous, non-cell derived, hydrogen peroxide formation. Using the phenol red assay hydrogen peroxide concentration was estimated in cell culture medium containing BNIPSpd. Catalase incubation was used to confirm the presence of hydrogen peroxide in control and 50μM BNIPSpd incubations, reducing the absorbance attributed to resorufin formation to 1% and 5% of non-catalase treatment values respectively. Data are means (n=3). *$P<0.05$ v. DMSO control

Figure 1 Bestwick et al.

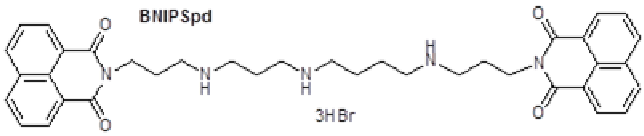


Figure 2 Bestwick et al.

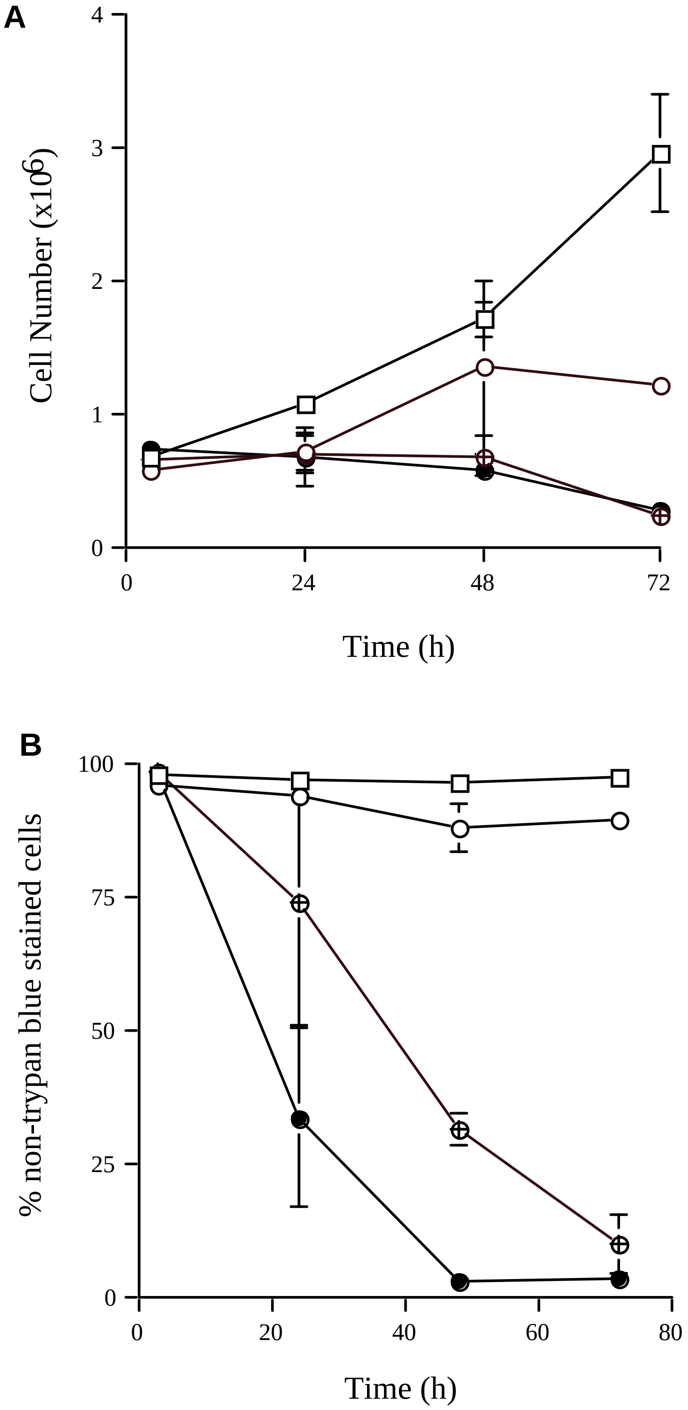
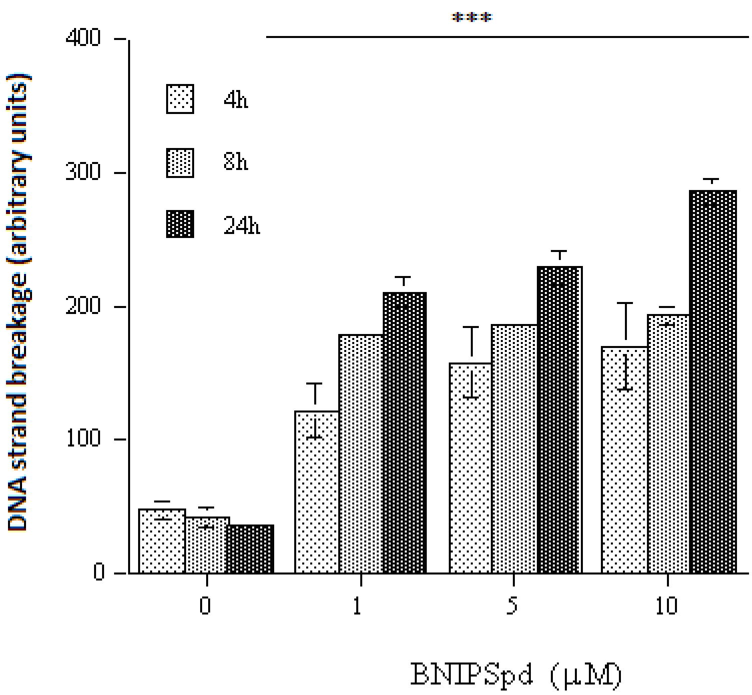


Figure 3 Bestwick et al.



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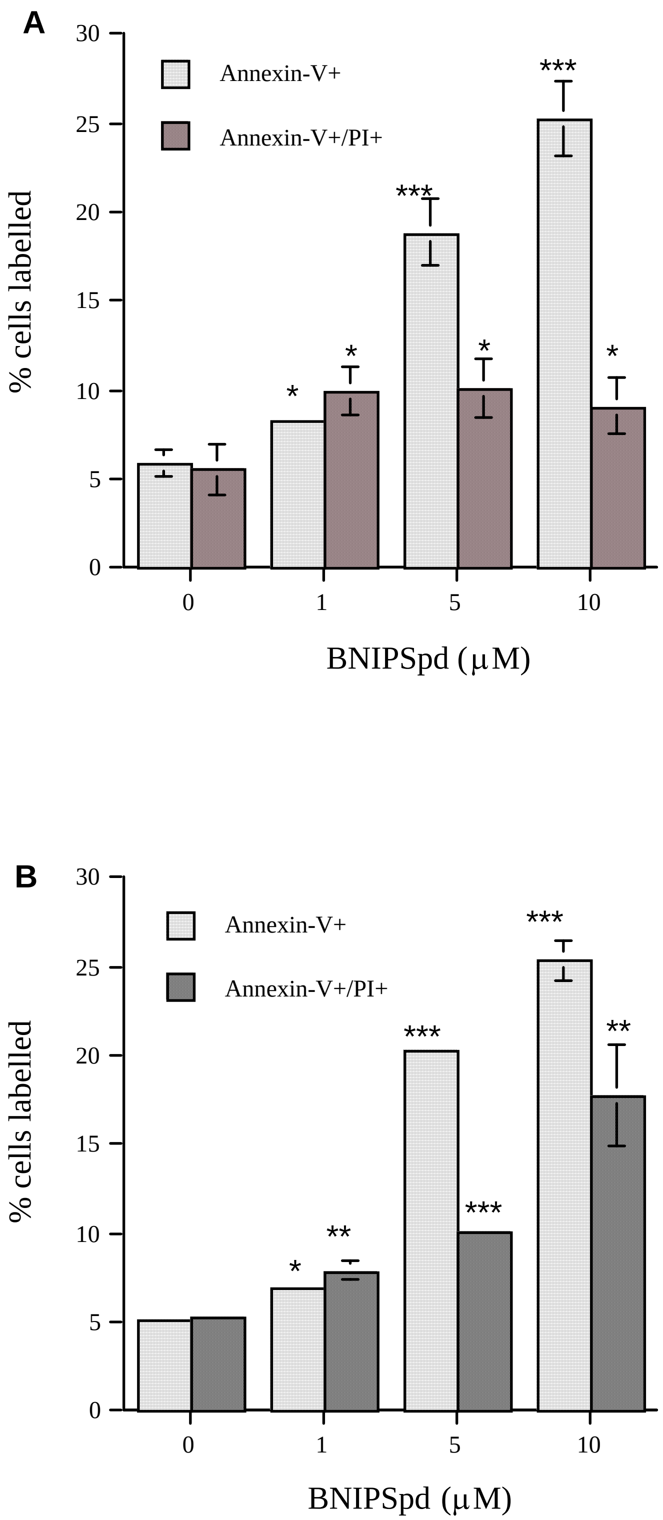

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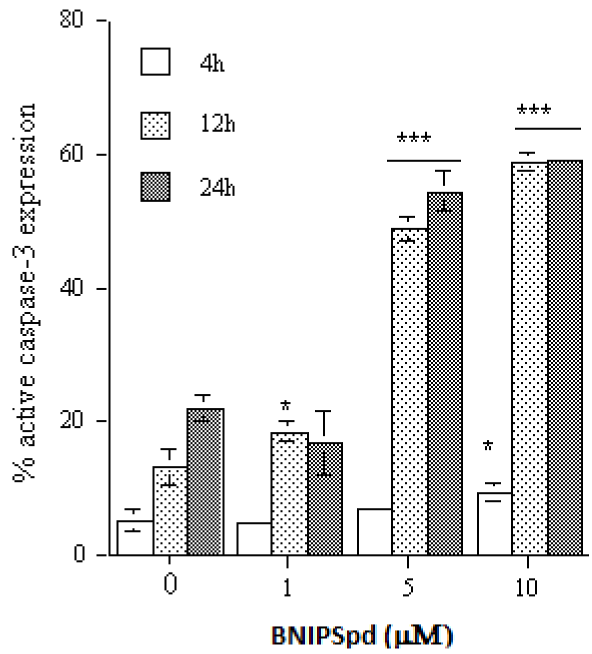
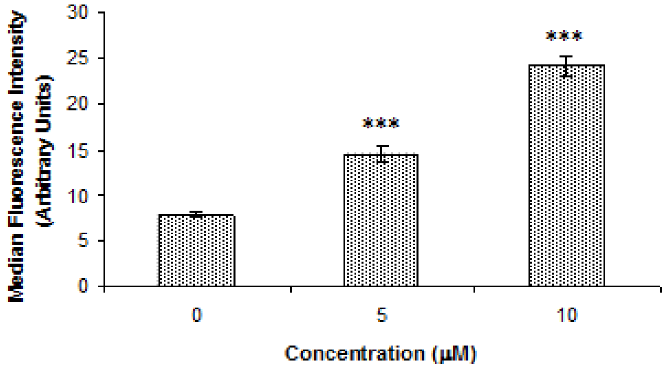


Figure 6 Bestwick et al.

A



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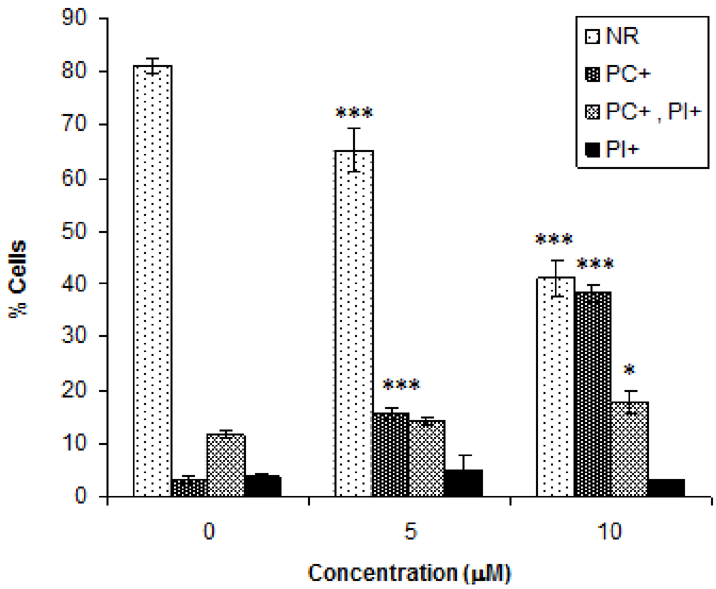
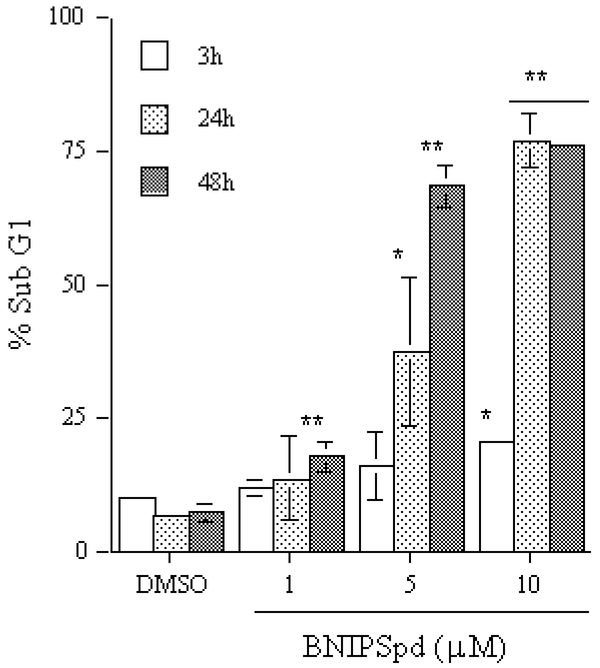


Figure 7 Bestwick et al

A



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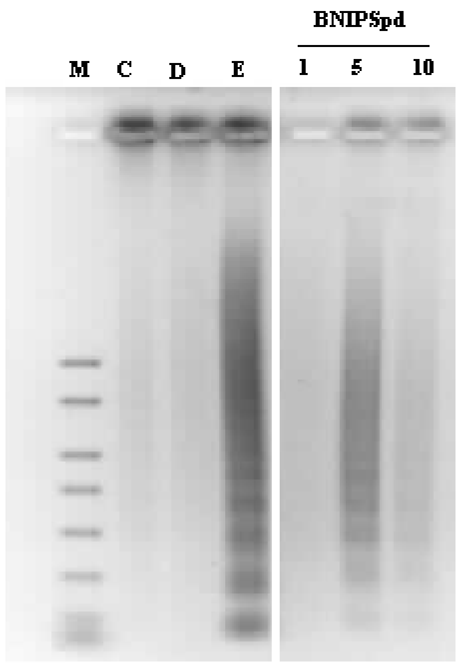

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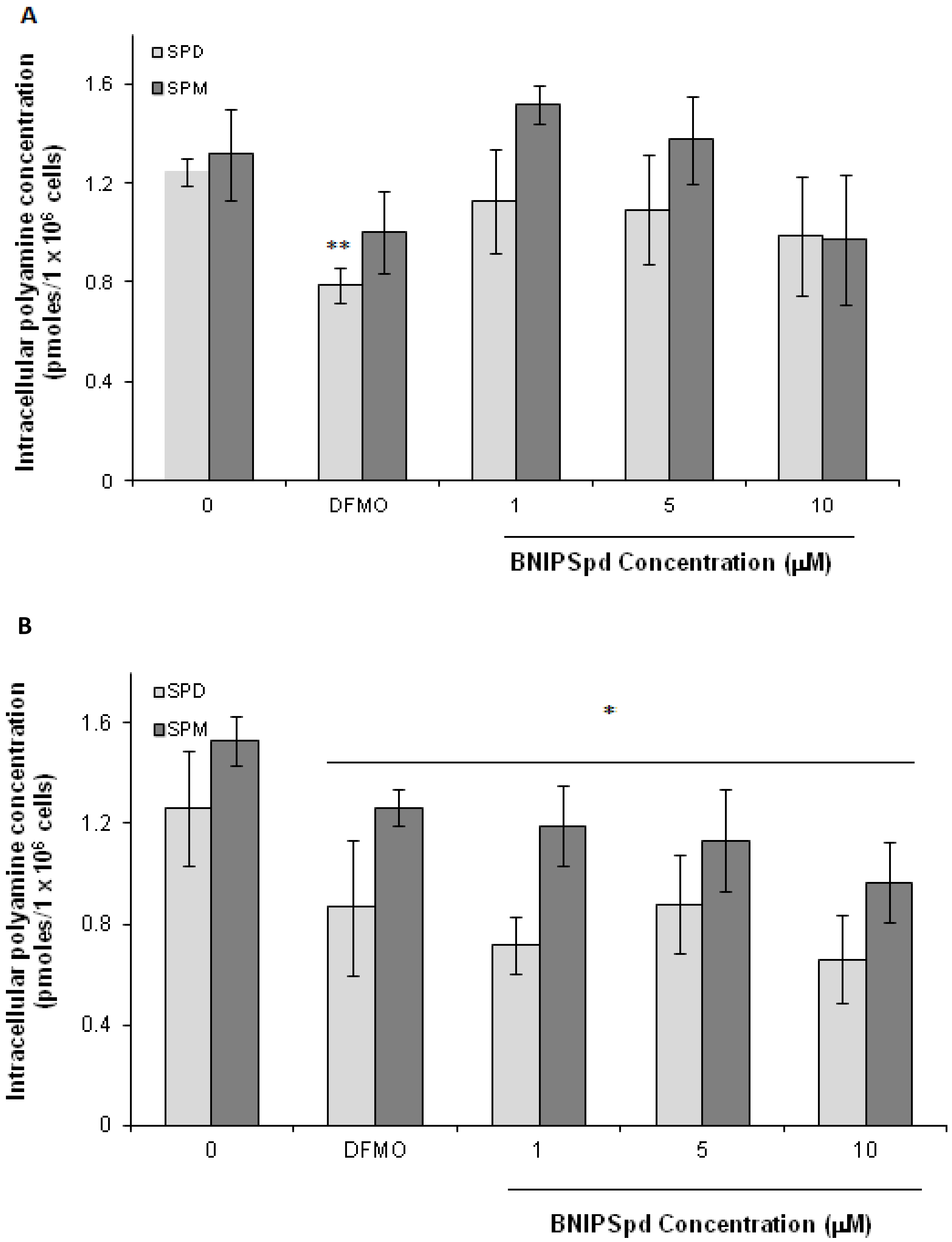

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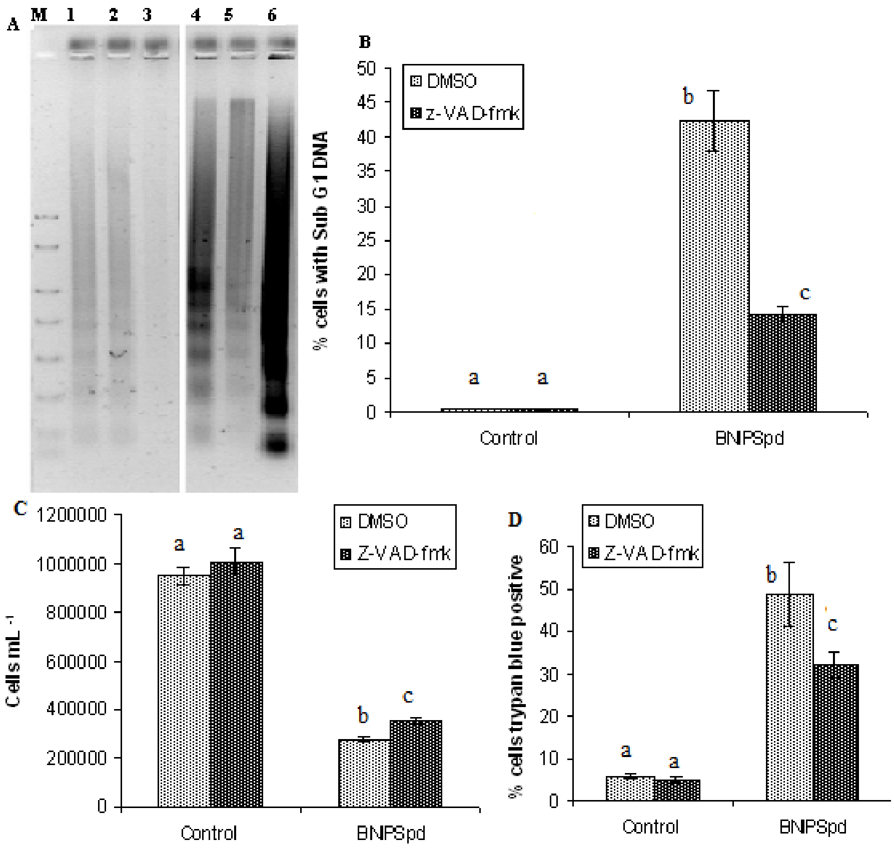

Figure 10 Bestwick et al