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

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ABSTRACT

Bisnaphthalimides are DNA intercalators of potential use as chemotherapeutics but for which the range of mechanism of action is only gradually being elucidated. Using human promyelocytic HL-60 cells, we extend characterization of the cytotoxicity of bisnaphthalimidopropylspermidine (BNIPSpd) and examine the relationship with caspase-activity. Within 4 h exposure, BNIPSpd (1–10 μM) induced significant DNA strand breakage. Evidence of apoptosis was progressive through the experimental period. Within 6 h, BNIPSpd increased the proportion of cells exhibiting plasma membrane phosphatidylserine exposure. Within 12 h, 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. 1 μM BNIPSpd exposure also increased hypodiploid DNA content by 48 h. Polyamine levels decreased by 24 h BNIPSpd exposure. The pan-caspase inhibitor, z-VAD-fmk, significantly decreased DNA degradation (hypodiploid DNA and DNA ladders) and cytotoxicity. 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.

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 and Xu, 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; Graça 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 and Jiang, 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 and Pavlov, 2000; Pavlov et al., 2001). As neoplastic transformation can be accompanied by elevated polyamine levels resulting from altered biosynthesis, catabolism and uptake

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

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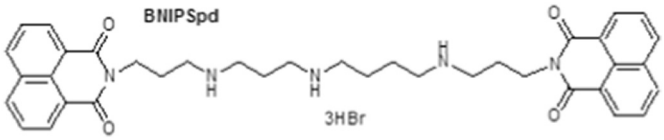

Fig. 1. Structure of Bisnaphthalimidopropylspermidine (BNIPSpd).

(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 (Berkshire, 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 (5×10^5 cells mL^{-1}) were incubated with 0.1-10 μM 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 $\mu g mL^{-1}$ 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