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MORPHOLOGICAL CHANGES IN MCF-7 HUMAN BREAST CANCER CELLS IN RESPONSE TO BIS-NAPHTHALIMIDOPROPYLSPERMIDINE-TREATMENT

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ABSTRACT

The effects of bis-naphthalimidopropylspermidine (BNIPSpd), a representative of a novel class of polyamine derivatives with antiproliferative properties on the morphology of MCF-7 human breast cancer cells were studied. BNIPSpd was shown to cause cytoplasmic shrinkage, observed by monitoring the cells by means of phase-contrast microscopy. Chromatin condensation and nucleolar disintegration were also detected by Hoechst 33342 and Giemsa staining the cells, treated with 10 μM BNIPSpd for 8h. Taken together the morhological changes observed strongly suggested that the growth inhibition of MCF-7 cells in response to BNIPSpd-treatment is realized via an induction of apoptosis. Moreover, nucleolus appears to be an important target in the mode of cytotoxic action of this novel polyamine derivative.

Introduction

Based on the known roles of natural polyamines spermine, spermidine and putrescine in cell growth and cell death, polyamine metabolism has attracted a great interest as a potential target of therapeutic interventions (1-3). Different types of structural analogues, homologues and derivatives of natural polyamines have been synthesized and these compounds have been shown to possess antiproliferative and anticancer properties (3-7). The ability of some synthetic polyamines to cause apoptosis as a specific type of cell death, in different cell lines has also been demonstrated (3, 8, 9). Apoptosis is the most predominant form of physiological cell death and diseases linked with suppression of apoptosis include cancer and some autoimmune disorders and viral infections. Hence the efforts aimed at treating these diseases by manipulating cell suicide mechanism would seem to have a great potential. Apoptosis could be distinguished on a morphological basis by contrast with another type of cell death, necrosis (10, 11). While the necrotic cell swells, the apoptotic cell visibly shrinks and adherent cells round up. Morphological changes, typical for appoptosis occur in nucleus where chromatin condenses and forms aggregates.

We have recently reported the synthesis and the anticancer potential of a series of bis-naphthalimidopropyl polyamine derivatives (12). These novel compounds were shown to possess high cytotoxic activity

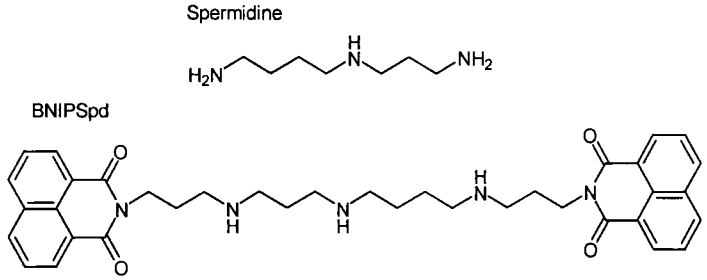


Fig. 1. Structures of spermidine and BNIPSpd .

towards MCF-7 human breast cancer cells and to bind to calf thymus DNA (13). The novel bisnaphthalimidopropyl polyamine derivatives were localized preferentially inside the nuclei of MCF-7 cells (13). These results indicated that the cytotoxic activity of the bis-naphthalimidopropyl polyamines may be at least in part, caused by their effects on DNA.

In this report we show some morphological changes in MCF-7 cells treated with one of the bisnaphthalimidopropyl polyamine compounds, i.e. bis-naphthalimidopropylspermidine (BNIPSpd) (**Fig. 1**). The morphological changes observed strongly indicate that the mechanism of the cytotoxicity of BNIPSpd in MCF-7 cells involves an induction of apoptosis.

Materials and Methods

Chemical Synthesis

BNIPSpd was synthesised as reported previously (12). The chemical structure and purity of the synthesised compound were determined by NMR, thin-layer chromatography and mass spectrometry.

Cell culture

MCF-7 cells were maintained in Earle's Minimal Essential Medium (Labtech Int., UK), supplemented with 10% fetal calf serum (Labtech Int., UK), 2 mM L-glutamine (Sigma), 1% non-essential amino acids (Sigma), 100 IU/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma). Exponentially growing cells were plated at 2.0 x 10^4 cells/cm^2 into 96-well plates and incubated for 24h before the addition of drugs.

Drug-treatment and cytotoxic assay

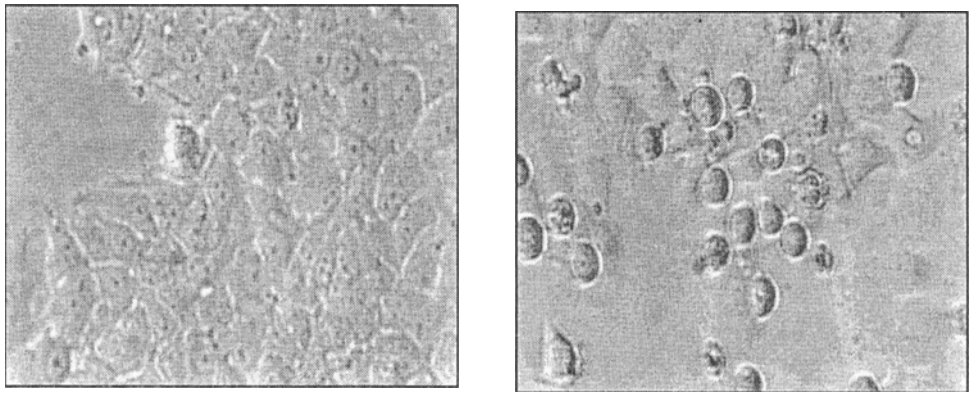
Stock solutions of compounds were initially solubilised in 10% dimethyl sulphoxide (DMSO) (Sigma) and further diluted in a fresh complete medium. The growth-inhibitory effects of the BNIPSpd were measured using a standard tetrazolium MTT assay (14) with minor modifications. After drug incubation in the range 10 μM - 100 μM at 37° C the medium was removed and 200 μl of MTT reagent (0.5 mg/ml) in serum free medium was added to each well. The plates were incubated at 37° C for 4h. At the end of the incubation, the medium was removed and the formazan crystals formed by MTT metabolism were solubilised by the addition of 200 μl of dimethyl sulphoxide to each well. The cellular metabolism of MTT was then quantified by reading the absorbance of the solubilised product at 560 nm on a microplate reader (Dynex Technologies, USA).

Assessment of cell shape and morphology

Cells were monitored by phase-contrast microscopy. Treated and untreated (control) cells were viewed using an inverted phase-contrast microscope model (PIM-II, World Precision Instruments Inc, Sarasota, USA and photographed using a Nikon camera.

Assessment of nuclear morhology

For the study of nuclear morphology exponentially growing cells were trypsinised and inoculated at a density of 2 x 10^4 cells/cm^2



A B

Fig. 2. Phase-contrast photomicrographs of untreated (A) and treated with 10 μM for 8h (B) MCF-7 cells. Representative pictures of two separate experiments.

onto sterile coverslips in 60 mm dishes (Nunc). After attachment the cells were incubated in the presence or absence of 10 μM polyamine analogue for different time intervals. At the desired time the medium was aspirated and the cultures were rinsed with PBS before adding methanol to fix the cells for 5 min. The fixed cells were stained with either Hoechst dye 33342 (Sigma) (0.1 mg/ml solution in PBS) or Giemsa (BDH Lab Suppl. UK) (10% solution in Sörensen phosphate buffer, pH 6.8). The coverslips were mounted onto microscope slides using liquid paraffin and PPX (BDH Lab suppl., UK) respectively. Hoechst (under UV excitation) and Giemsa stained nuclei were visualized and photographed using a Leica DMRB microscope (Leica Microsystems Holdings Gmbh, Germany).

Results and Discussion

BNIPSpd was recently found to possess the highest growth-inhibitory activity towards MCF-7 cells among the bis-naphthalimidopropyl polyamine derivative tested (13) with an IC_{50} value of 1.38 μM after 48h of drug incubation. Therefore, we chose this compound as a representative to investigate the effects of the novel polyamine derivatives on MCF-7 cells. Although the present study focuses on the morhological changes in MCF-7 cells caused by BNIPSpd treatment, MTT assay was carried out in parallel to evaluate the compound cytotoxicity.

The cytotoxicity of BNIPSpd (confirmed by MTT assay) was accompanied with apparent cell shrinkage, which was clearly detectable following even the short-term incubation period of 8h with 10 μM of the compound (**Fig. 2**). It is well known that cytoplasmic shrinkage represent one of the first events in cell death via apoptosis (10). Apoptosis is known to be involved as in the removal of redundant and unwanted cells during normal development, as well as in the death of cancer cells caused by the action of various agents (15). Besides cytoplasmic shrinkage, the chromatin condensation and nuclear fragmentation are other recognized morphological changes that occur during apoptotic progression (10). Therefore, we decided to further investigate the ability of BNIPSpd to induce apoptosis in MCF-7 cells by staining the cells with the fluorescent die Hoechst 33342, which allows the evaluation of the nuclear morphology. Numerous cells with dense, pyknotic nuclei (the brighter fluorescence) (**Fig. 3, B**), were observed in treated, but not in control MCF-7 cultures (**Fig. 3, A**). Another intriguing observation was that the nucleoli, clearly shaped as

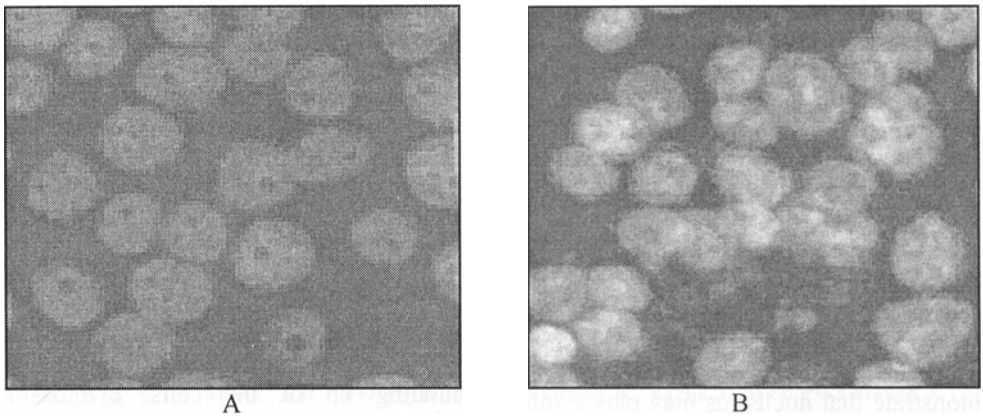


Fig. 3. Photomicrographs of untreated (A) and BNIPSpd-treated (B) MCF-7 cells. Cells were incubated with the drug (10 μM) for 8 h, fixed in methanol and stained with Hoechst 33342. Representative pictures of three separate experiments.

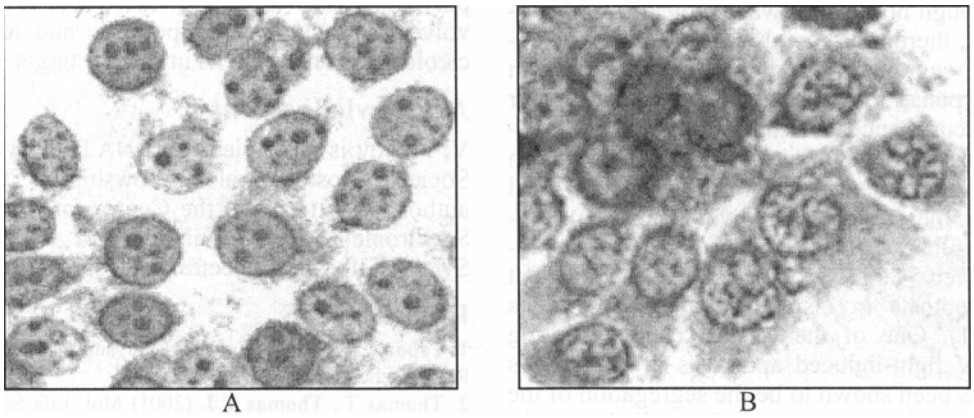


Fig. 4. Photomicrographs of untreated (A) and BNIPSpd-treated (B) MCF-7 cells. Cells were incubated with the drug (10 μM) for 8h, fixed in methanol and stained with Giemsa. Representative pictures of three separate experiments.

darker dots inside the nuclei of untreated cells (Fig. 3, A) were not longer visible in the nuclei of treated cells (Fig. 3, B). Since it was worth further studying the disintegration of nucleoli in response to BNIPSpd-treatment, we stained the cells with Gimsa dye. Under the light microscope nucleoli were clearly visible as dark dots inside the Gimsa-stained nuclei of the control cells (**Fig. 4, A**), but not in the nuclei of treated cells (**Fig. 4, B**).

A recognized goal in cancer therapy is provoking apoptotic death in cancer cells (15). Since breast cancer is among the notorious neoplastic diseases, the design of novel drugs, capable of effectively inhibiting the growth of breast cancer cells via apoptosis represents an important initial step of breast cancer treatment strategies. MCF-7 cells have been used in the evaluation of the biochemical properties of many anticancer compounds, including polyamine analogues (8, 16). We report in this paper that BNIPSpd, that represents a new type

of antiproliferative synthetic polyamines causes morphological changes in MCF-7 cells, which indicate an induction of apoptosis. Apart from the cytoplasmic shinkage and chromatin condensation, another intriguing observation was the "disappearance" of the nucleoli as a result of BNIPSpd-treatment.

It is well-known that nucleolus is the site of ribosomal gene transcription, rRNA processing and pre-ribosomal particle assembly (17). Moreover, recent data, summarized in the review of Olson et al.(18) demonstrate that nucleolus may play a role in nuclear export, sequestering regulatory molecules, modifying small RNSs, assembling ribonucleoproteins and controling aging. Although nucleolar disintegration is though not to be always typical for apoptosis, there are several examples that nucleolar segregation and disassembly occur in response to apoptogenic agents and under circumstances of inhibition of RNA synthesis (18, 19). For example, disintegration of nucleolus has been shown to be typical for the apoptosis in thymocytes (20). Nucleolus has also been shown to be a preferred target for caspase 3-dependent apoptosis in cisplatin-treated HeLa cells (21). One of the earliest changes during UV light-induced apoptosis in HeLa cells has been shown to be the segregation of the nucleolar components into distinct subcompartments (18). Similar events can be observed to occur in BNIPSpd-treated MCF-7 cells. As stated above, it was previously shown that the novel bis-naphthalimidopropyl polyamine derivatives were capable of strongly stabilizing calf thymus DNA duplex and thus, it might be suggested that these novel compounds could negatively affect DNA–opening processes such as RNA synthesis. Taken together our previous results and the present observation that BNIPSpd causes nucleolar disintegration suggest that these novel compounds presumably target nucleolar DNA. BNIPSpd might affect nucleolar DNA by a manner that leads to nucleolar disintegration accompanied by nucleolar disfunction (including an inhibition of ribosomal RNA synthesis etc.).

Additional studies on the underlying mechanisms of BNIP polyamine derivative-caused apoptosis would contribute to further developing the anticancer potential of these novel compounds.

Conclusions

Our results demonstrate that the treatment of MCF-7 cells with BNIPSpd causes rounding up of the cells, cytoplasmic shrinkage, nuclear condensation and nucleolar disintegration. Based on these morphological observations it could be suggested that the cytotoxicity of this polyamine derivative in MCF-7 cells involves an induction of apoptosis and nucleolus is presumably its important target.

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