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Bisnaphthalimidopropyl diaminodicyclohexylmethane induces DNA damage and repair instability in triple negative breast cancer cells *via* p21 expression

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Abstract

Bisnaphthalimidopropyl diaminodicyclohexylmethane (BNIPDaCHM) bisintercalates to DNA and is a potential anti-cancer therapeutic. In an attempt to elucidate the mechanism(s) underlying the potential of BNIPDaCHM; earlier work was extended to investigate its effect on DNA damage and repair as well as cell cycle modulation, in a triple negative breast cancer (TNBC) cell line *in vitro*.

BNIPDaCHM significantly decreased cell viability in a concentration (≥ 5 μM) and time (≥ 24 hours) dependent manner. The mechanism of this growth inhibition involved alterations to cell cycle progression, an increase in the sub-G1 population and changes to plasma membrane integrity/permeability observed by flow cytometry and fluorescence microscopy with acridine orange/ethidium bromide staining. Using single cell gel electrophoresis (Comet assay) and fluorescence microscopy to detect γ-H2AX-foci expression; it was found that after 4 hours, ≥ 0.1 μM BNIPDaCHM treatment-induced significant DNA double strand breaks (DSBs). Moreover, exposure to a non-genotoxic concentration of BNIPDaCHM induced a significant decrease in the repair of oxidative DNA strand breaks induced by hydrogen peroxide. Also, BNIPDaCHM-treatment induced a significant time dependent increase in $p21^{Waf/Cip1}$ mRNA expression but, did not alter p53 mRNA expression.

In conclusion, BNIPDaCHM treatment in MDA-MB-231 cells was associated with a significant induction of DNA DSBs and inhibition of DNA repair at non-genotoxic concentrations *via* p53-independent expression of $p21^{Waf1/Cip1}$. The latter may be a consequence of novel interactions between BNIPDaCHM and MDA-MB-

231 cells which adds to the spectrum of therapeutically relevant activities that may be exploited in the future design and development of naphthalimide-based therapeutics.

Keywords

Bisnaphthalimidopropyl; DNA damage; DNA repair; p21 expression; Triple negative breast cancer

List of Abbreviations

Acridine orange (AO); Bisnaphthalimidopropyl (BNIP); Bisnaphthalimidopropyl diaminodicyclohexylmethane (BNIPDaCHM); Breast cancer (BC); 4',6-diamidino-2-phenylindole dihydrochloride (DAPI); Double strand breaks (DSBs); Dimethyl sulfoxide (DMSO); Estrogen receptor (ER); Ethidium bromide (EtBr); Foetal calf serum (FCS); Human epidermal growth factor receptor (HER2); Hydrogen peroxide (H_2O_2); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); Phosphate buffered saline (PBS); Phosphatidylserine (PS); Progesterone receptor (PR); Propidium iodide (PI); Single cell gel electrophoresis (SCGE); Triple negative breast cancer (TNBC).

1. Introduction

Breast cancer (BC) is the most frequently diagnosed cancer, as well as, the leading cause of death in the female population [1]. Triple negative breast cancer (TNBC) accounts for 12-20% of all BCs and, in this phenotype of BC, cells lack the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2/*neu*) [2, 3]. Drug-resistant TNBCs metastasise rapidly, have poor prognosis which renders them extremely difficult to treat and, they also appear to be occurring more frequently in younger women [3, 4]. Therefore, it is crucial to develop and test new, selective and efficient anti-cancer drugs that counteract TNBC cell growth, but also limit side effects which are often unmanageable for patients.

Chemotherapy, using a broad spectrum of cytotoxic drugs, such as, doxorubicin, daunorubicin, or epirubicin, is the primary treatment offered to TNBC patients. The therapeutic use of these drugs relies on their ability to interact and target DNA by intercalation [5] thus, interfering with transcription and/or replication. Notably, this mode of action remains the most effective treatment for, not only breast cancer, but also acute leukaemia, ovarian and bladder cancers [6].

Bisnaphthalimides constitute a promising group of anti-cancer compounds which target DNA by intercalation and have strong cytotoxic properties observed *in vitro* [7, 8, 9]. Typically, these compounds consist of two naphthalamido-chromophore units linked by alkyl chains containing at least one amino group [10]. Noteworthy examples are Elinafide (LU79553) [11, 12] and Bisnafide (DMP-840) [13] which progressed to Phase I and II clinical trials for advanced solid cancers. Despite encouraging preliminary anti-tumour activities, problems with efficacy and

adverse dose-limiting toxicities, for example, myelosuppression [11, 13] and neuro-muscular toxicity [12] of a cumulative nature were observed. To address such issues, more research is required in order to improve where these former bisnaphthalimide derivatives have clinically failed.

For a number of years, our research group have been designing and synthesising bisnaphthalimidopropyl (BNIP) derivatives linked to natural polyamines [14, 15, 16], and to a selection of diamino and triamino alkyl chains [17, 18, 19, 20] which have enhanced aqueous solubility and strong DNA-binding affinities. BNIP derivatives were found to be more cytotoxic (IC_{50} values in the range of 4.9-12.7 μM) than the chemotherapy drug doxorubicin (IC_{50} value 14.4 μM) in breast cancer MDA-MB-231 cells, after 24 hours [20]. Moreover, BNIP derivatives were less cytotoxic in non-tumourigenic breast epithelial MCF-10A cells than doxorubicin; which gave an indication of the selectivity of BNIP derivatives in both cancerous and surrounding healthy tissues [20].

DNA was suggested to be a potential target for BNIP derivatives by Barron *et al.* [20]; however, the specific mechanism by which DNA damage is induced has not been widely investigated. Recent alterations to the linker chain confirmed BNIP moiety is crucial for activity [20, 21]. Novel modifications to the linker chain, such as, introduction of a bicyclohexylmethane group (bisnaphthalimidopropyl diaminocyclohexylmethane; BNIPDaCHM) (Fig. 1.) - reduced the flexibility of the linker - but enhanced cellular uptake and biological activity *in vitro* against MDA-MB-231 cells [20]. Even though DNA is considered to be intrinsic to bisnaphthalimide derivative efficacy as observed in colon cancer cells [8, 22, 23, 24]; the relationship between bisnaphthalimide exposure and DNA stability in BC cells, particularly TNBC cells, has not been widely investigated.

This study aims to investigate, for the first time, the effect of BNIPDaCHM on DNA stability and repair, as well as, cell cycle modulation in one of the most commonly studied TNBC cell line (i.e., MDA-MB-231 cells) *in vitro*. These cells were selected to extend our previous work [20] and, also because the treatment of TNBC is associated with poor outcome and still lacks the benefit of targeted therapy. Therefore, treatment of TNBC is a significant clinical problem for which there is currently not an effective treatment option and so, investigating the effect of a potentially promising anti-cancer derivative in a TNBC cell line may address an important area of cancer research.

2. Materials and Methods

2.1. Materials

All chemicals and cell culture reagents were purchased from Sigma-Aldrich Ltd (Poole, UK) unless otherwise stated. Sterile, disposable cell culture plastics were obtained from Nunc (Fisher ThermoScientific, Loughborough, UK). BNIPDaCHM was synthesised and characterised as reported in Barron *et al.* [20]. Stock BNIPDaCHM (10 mM) was prepared in 50% (v/v) dimethyl sulfoxide (DMSO), stored at 4°C and diluted to the desired final concentrations in growth media containing serum.

2.2. Cell culture

Human breast epithelial MDA-MB-231 cancer cells were purchased from the European Collection of Cell Cultures (ECACC Catalogue No.: 92020424, Lot. No. 05K032) (Public Health England, UK). MDA-MB-231 cells were maintained in RPMI-1640 medium (containing GlutaMAX™-1 with 25 mM HEPES) supplemented with 10% (v/v) foetal calf serum (FCS) and 1% (v/v) penicillin (100 U/mL)/streptomycin (100 µg/mL).

MDA-MB-231 cells were incubated at 37°C in a 5% CO_2 air humidified atmosphere. Cells were media-changed and sub-cultured appropriately and all washes were performed with phosphate buffered saline (PBS).

MDA-MB-231 cells were seeded in 96 well plates (8 x 10^3 cells/well) for cytotoxic studies; 24 well plates (8 x 10^4 cells/well) for DNA damage (strand breakage), DNA repair and acridine orange/ethidium bromide staining; 6 well plates (4 x 10^5 cells/well) for annexin V conjugate labelled with fluorescein isothiocyanate

(Annexin V-FITC) flow cytometry experiments and 25 cm^2 flasks (3.6 x 10^4 cells/cm^2) for cell cycle analysis and RNA expression. Cells were allowed to adhere overnight. For the γ-H2AX-foci detection by fluorescent microscopy, MDA-MB-231 cells were cultured overnight in 12-well plates to 70-80% confluency prior to treatment.

DMSO, with or without BNIPDaCHM, was added to the monolayers at a volume not exceeding 0.5% (v/v) solvent, so that the final concentration of BNIPDaCHM was ≤ 5 μM when used in the DNA strand breakage, DNA repair, fluorescence microscopy, flow cytometry and RT-PCR experiments as detailed below. DMSO-treated cells were denoted as the control in all experiments.

2.3. Cytotoxicity – MTT assay

BNIPDaCHM cytotoxicity was quantified using the standard colourimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [25]. MDA-MB-231 cells were incubated with BNIPDaCHM (0-40 μM; for 4-72 hours) in 96 well plates. After the desired incubation time, culture medium was removed, 100 μL MTT (1 mg/mL) solution added and incubated at 37 °C for 4 hours. The metabolised MTT product was dissolved in DMSO (100%, 200 μL) and quantified by measuring the absorbance at 560 nm in a plate reader (Bio-Tek, UK). The mean absorbance values for the negative control (DMSO-treated cells) were standardised as 100% (i.e., no growth inhibition) and the results for BNIPDaCHM expressed as % absorbance of DMSO-treated cells. The IC_{50} is the concentration of BNIPDaCHM required to inhibit cell growth by 50% of DMSO-treated cells.

2.4. Cell cycle analysis – Flow cytometry

Cell cycle progression was determined in MDA-MB-231 cells incubated with BNIPDaCHM (5 µM) or etoposide (10 µM; positive control) for 24 hours, at 37 °C. Medium was removed and collected and, cells washed twice with PBS. Both washes were collected and the cells detached using trypsin. The equivalent cells and washes were centrifuged at 2,500 rpm for 5 minutes, at 4 °C. Cell pellets were washed in PBS (1 mL) and centrifuged as before. The resultant cell pellets were resuspended in 100 µL PBS and fixed with 70% (v/v) ice-cold ethanol (900 µL) at -20 °C for 2 hours. The cell pellets were centrifuged at 5,000 rpm for 5 minutes, at 4 °C. As before, cell pellets were washed in PBS (1 mL) and re-centrifuged at 5,000 rpm for 5 minutes, at 4 °C. The cell pellet was resuspended in 500 µL PBS/DNA (1:1) extraction buffer (0.2 M Na_2PO_4, 4 mM citric acid, pH 7.8) and incubated for 5 minutes, at room temperature (RT). The extract was centrifuged at 5,000 rpm for 5 minutes, at 4 °C; the supernatant removed and the cell pellet resuspended in 500 µL DNA staining solution (0.2 mg/mL ribonuclease A (DNAse-free) and 20 µg/mL propidium iodide (PI)) and incubated for 30 minutes, at 4 °C, in the dark. PI stained nuclei were analysed by the Epics XL-MCL flow cytometer (Beckman-Coulter, UK). A total of 10,000 events were acquired with EXPO32 ADC XL 4 color and EXPO32 ADC analysis software (Beckman Coulter, UK). The percentage of cells with DNA content in sub-G_1, G_1, S and G_2/M were calculated from histograms of linear FL-2 plots (575 nm) in the gated region.

2.5. Acridine orange/ethidium bromide staining – Fluorescence microscopy

MDA-MB-231 cells were incubated with BNIPDaCHM (0-5 µM; for 0.5, 6 and 24 hours, at 37 °C) or etoposide (10 µM; for 24 hours, at 37 °C; positive control).

Medium was removed and cells washed twice with PBS. Changes to cell morphology, plasma membrane integrity and nuclear structure were assessed by acridine orange/ethidium bromide (AO/EtBr) staining (40 µL of 100 µg/mL solution to 1 mL PBS) using fluorescence microscopy (Leica DMIL fluorescence microscope (Leica Microsystems, Milton Keynes, UK) with a UV filter (excitation bandpass 450-490 nm and emission longpass 515 nm) and Leica DC 200 camera (Leica Microsystems, Milton Keynes, UK).

2.6. Detection of apoptosis - Flow cytometry

Detection of apoptotic cells, phosphatidylserine (PS) exposure and cell membrane integrity/permeability of BNIPDaCHM-treated MDA-MB-231 cells (0-5 µM; for 0.5 hours, at 37 °C) were determined *via* Annexin V-FITC in conjunction with 7-amino actinomycin D (7-AAD) staining kit according to manufacturers instructions (Beckman Coulter, High Wycombe, UK). Briefly, cell pellets were resuspended in 100 µL ice-cold Binding Buffer (1x) with 10 µL Annexin V/FITC solution and 20 µL 7-AAD viability dye subsequently added. Pellets were mixed gently and incubated on ice for 15 minutes in the dark. Ice-cold binding buffer (1x; 400 µL) was added, mixed gently and analysed using Epics XL-MCL flow cytometer (Beckman-Coulter, UK); with a total of 10,000 events acquired. Formaldehyde (3% v/v) was used as an experimental control to configure the gate settings due to its ability to induce maximal apoptotic cell death after 30 minutes. The FL-1 (525 nm) channel was gated for annexin V-FITC and FL-4 (675 nm) channel gated for detection of 7-AAD. The percentage of apoptotic and necrotic cells were analysed from histogram plots of LOG FL-1 (525 nm) and LOG FL-4 (675 nm), respectively.

2.7. DNA damage (strand breakage) – Comet assay

MDA-MB-231 cells were incubated with BNIPDaCHM (0-10 μM; for up to 24 hours, at 37 °C) or positive controls, camptothecin (10 μM for 2 hours, at 37 °C) or hydrogen peroxide (H_2O_2; 200 μM for 5 minutes, on ice). Human breast epithelial MCF-10A cells (purchased from the American Tissue Culture Collection (ATCC Catalogue No.: CRL-10317, Lot. No.: 3982356) are non-tumourigenic and were used as a normal breast cell line control. Cells were collected using trypsin and DNA damage assessed using single-cell gel electrophoresis (SCGE; Comet assay) under alkaline conditions as previously described by Singh *et al.* [26] and further adapted in Dance *et al.* [16] and Ralton *et al.* [19]. Quantification of DNA strand breaks was determined visually by fluorescence microscopy (Leica DMRB fluorescence microscope (Leica Microsystems, Milton Keynes, UK)) with D filter (excitation bandpass 355-425 nm and emission longpass 470 nm) and classified in order of the degree of DNA strand breaks, assessed by the relative intensity of fluorescence in the comet head and the length of the comet tail, and given a score of 0, 1, 2, 3 or 4 (from undamaged, 0, to maximally damaged, 4) [19]. One hundred comets were scored blind per sample, and hence, the total score of each sample can range from 0 (all undamaged) to 400 (all maximally damaged) [27].

2.8. γ-H2AX-foci expression – Fluorescence microscopy

MDA-MB-231 cells were incubated with BNIPDaCHM (0-5 μM; for up to 24 hours, at 37 °C) or camptothecin (10 μM for 2 hours, at 37 °C; positive control). The medium was replaced and the cells left to recover for 2 hours. The medium was then removed and the cells washed 3 times with PBS. The cells were subsequently fixed with 4% (w/v) paraformaldehyde (Wako Pure Chemical Ind., Ltd, Osaka, Japan) for

30 minutes at RT. The cells were washed as before and permeabilised for 30 minutes with 0.1% (w/v) Triton-X in PBS (PBST) at RT. The cells were blocked with 5% blocking one buffer (BOB; Nacalai, Kyoto, Japan) in PBST for 30 minutes at RT. γ-H2AX monoclonal antibody (clone JBW301; Millipore, Temecula, USA) was added at 1:200 in 5% BOB-PBST for 1 hour at RT. The cells were washed as previously described and the Alexa Fluor 488 goat anti-mouse IgG antibody (Invitrogen, Carlsbad, CA, USA) added at 1:200 for 1 hour at RT. The cells were washed as before and the samples mounted with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) Fluoromount-G® (Southern-Biotech, Birmingham, AL, USA) and a glass slide placed over each sample. The cells were viewed using an Olympus IX71 microscope: DAPI (excitation 330-385 nm and emission 420 nm) and for γ-H2AX-foci (excitiation 470-495 nm and emission 510-550 nm). Images from one microscope field of view per sample were taken and analysed using QCapture Pro 6.0 (QImaging, BC, Canada). γ-H2AX stained nuclei and DAPI stained nuclei (i.e., total number of cells) were counted. The γ-H2AX stained nuclei/DAPI stained nuclei ratio was calculated and expressed as a percentage [28].

2.9. DNA repair of hydrogen peroxide-induced DNA strand breakage – Comet assay

MDA-MB-231 cells were incubated with BNIPDaCHM (nontoxic concentrations of 0.1 or 1 µM for 4 and 24 hours, respectively). Cells were washed twice with PBS before incubation with H_2O_2 (200 µM for 5 minutes on ice) to induce oxidative DNA damage. After 5 minutes, the medium and H_2O_2 were removed, cells washed twice with PBS and fresh RPMI medium added. Cells were incubated for up

to 24 hours, at 37 °C after which cells were collected (i.e., 0, 2, 4, 8 and 24 hours after addition of RPMI medium) and processed for SCGE as previously detailed.

2.10. p53 and p21 gene expression – RT-PCR

Total RNA was extracted from BNIPDaCHM (5 μM) treated MDA-MB-231 cells, after 1 or 4 hours, using TRIzol (Invitrogen, Paisley, UK) according to manufacturer's instructions. Total RNA concentrations were quantified using a Hελios γ UV/Visible spectrophotometer (Thermo Electron Corporation, Madison, USA). Equal amounts of RNA (100 ng) were reverse-transcribed using 5x first strand buffer, dithiothreitol (DTT), deoxynucleotide triphosphate (dNTP's) (Promega, Madison, USA), recombinant ribonuclease inhibitor RNaseOUT™ (Invitrogen, Paisley, UK), bovine serum albumin (BSA) (New England Biolabs Inc, Hitchin, UK), random hexadeoxynucleotides (Promega, Madison, USA) and Superscript™ III reverse transcription (RT) (Invitrogen, Paisley, UK) using an *i*Cycler Thermal Cycler (Bio-Rad Laboratories, USA).

Expression of human p53, human $p21^{Waf1/Cip1}$ and β-actin (Table 1) were studied by semi-quantitative PCR using an *i*Cycler Thermal Cycler (Bio-Rad Laboratories, USA). The amplification conditions used were as follows: initial denaturation (4 minutes at 94 °C); followed by 25 cycles comprising of 1 minute at 94 °C, 2 minutes at 59 °C and 2 minutes at 72 °C for p53 and $p21^{Waf1/Cip1}$ or 25 cycles comprising 1 minute at 94 °C, 2 minutes at 68 °C and 2 minutes at 72 °C for β-actin; and 1 cycle of final extension (8 minutes at 72 °C). Amplification products were electrophoresed in 1.5% (w/v) agarose gels and visualised with ethidium bromide using a ChemiDoc EQ system (Bio-Rad Laboratories, Hemel Hempstead, UK) and

quantified using Quantity One (Version 4.5.0) software (Bio-Rad Laboratories, Hemel Hempstead, UK).

2.11. Statistics

Unless otherwise stated, each data set contained a minimum of two independent experiments, in which each experiment was comprised of at least 3 internal replicates. Data are presented as mean ± SEM and, where appropriate, data was compared by Student *t*-test (Graphpad Prism) or by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison post *t*-test (Graphpad Prism). $P<0.05$ was considered to be statistically significant.

3. Results

3.1. BNIPDaCHM treatment reduced cell viability

The cytotoxicity of BNIPDaCHM (0.01-40 μM) was investigated in MDA-MB-231 cells over 72 hours and quantified using the MTT assay (Fig. 2), where mitochondrial activity is assumed to reflect the rate of cell metabolism and, thus, the rate of enzymatic conversion is directly representative of cell viability. The percentage cell viability was normalised to DMSO-treated cells (i.e., control (0.2% (v/v) DMSO)).

BNIPDaCHM significantly decreased cell viability in a concentration (≥ 5 μM) and time (≥ 24 hours) dependent manner, compared to control cells. Cell viability was significantly decreased with 5 μM BNIPDaCHM; with a decrease of 21, 46 and 60% after 24, 48 and 72 hours, respectively (Fig. 2). The IC_{50} values were 6.8, 5.2 and 4.6 μM after 24, 48 and 72 hours, respectively (Table 2).

From these results, ≤ 5 μM BNIPDaCHM concentrations were used in all subsequent experiments in this study.

3.2. BNIPDaCHM treatment induces cell cycle instability and a loss of plasma membrane integrity

As increased proliferation is a characteristic common to tumour cells, which are more susceptible to cell cycle modulation, the effects of BNIPDaCHM treatment on the cell cycle of MDA-MB-231 cells was investigated using flow cytometry and PI staining (Figs. 3 and 4). PI is a DNA intercalator that can bind to DNA specifically following the loss of plasma membrane integrity; with the amount of PI proportional

to DNA content, which indicates the distribution of cells within the different cell cycle phases (Fig. 3A).

BNIPDaCHM (5 μM) treatment significantly increased (31%) the sub-G1 population (i.e., cells with low DNA content) and induced a significant decrease (22% and 8%) in the proportion of MDA-MB-231 cells in G1 and G2/M, respectively, compared to the control (DMSO-treated cells) (Fig. 3B). Cells in S phase were decreased, but not significantly, when compared to control cells (Fig. 3B). Treatment with etoposide (10 μM; positive control) increased (17%), the sub-G1 population but, not to the same extent as BNIPDaCHM. Also, cell arrest in S phase (8% increase) was observed. Thus, BNIPDaCHM and etoposide use differential mechanisms of action (Fig. 3B).

Cell cycle checkpoints represent an intersection of cell survival and death where conditions for successful interphase and mitosis have to be favourable for complete cell division or cell death is triggered. In accordance with this and the finding that BNIPDaCHM increased the cell population in sub-G1; apoptosis was examined by fluorescence microscopy and flow cytometry to further determine the effects of BNIPDaCHM on early cell death in MDA-MB-231 cells (Fig. 4).

The effect of BNIPDaCHM (1 or 5 μM; 0.5-24 hours) on chromatin changes in MDA-MB-231 cells relative to membrane integrity was demonstrated by fluorescence microscopy following AO/EtBr staining (Fig. 4A). Both AO and EtBr intercalate with DNA; AO fluoresces green in viable cells with intact membranes (i.e., viable and early apoptotic cells), whilst EtBr fluoresces orange in non-viable cells (i.e., late apoptosis and necrosis). EtBr can only be taken up in cells when plasma membrane integrity has been compromised. Membrane integrity was compromised with $\geq$ 1 μM BNIPDaCHM (Fig. 4A). After 0.5 hours, early apoptotic cells were

evident in a dose dependent manner in BNIPDaCHM-treated MDA-MB-231 cells, compared to control cells. After 6 hours, late apoptotic cells were evident; however, viable and early apoptotic cells were also present, in both control and BNIPDaCHM-treated MDA-MB-231 cells. After 24 hours, viable, early apoptotic and late apoptotic cells were evident in control and BNIPDaCHM-treated MDA-MB-231 cells. A visible loss of cell number and cell shrinkage was observed with 5 µM BNIPDaCHM, compared to control cells. Finally, a higher loss of membrane integrity (i.e., appearance of late apoptotic cells) was observed with etoposide (10 µM), compared to 5 µM BNIPDaCHM, after 24 hours (data not shown).

For the quantitative detection of early apoptotic cells, PS exposure and cell membrane integrity/permeability was determined by Annexin V-FITC. Annexin V-FITC specifically binds to exposed PS residues on the outer layer of the plasma membrane and hence stains early apoptotic cells. However, 7-AAD labels cells when plasma membrane integrity has been lost thus, necrotic cells. After 0.5, 4 and 6 hours, no significant difference in the percentage of cells labelled annexin V-FITC positive or 7-AAD was observed in 5 µM BNIPDaCHM-treated MDA-MB-231 cells, compared to control cells (Fig. 4B and 4C). However, a trend for increased annexin V-FITC and 7-AAD labelling in cells treated with BNIPDaCHM indicated a loss of plasma membrane integrity (Fig. 4C). These results correlate with the AO/EtBr staining observational study (Fig. 4A) but, no significant differences were observed.

3.3. Cell cycle instability is associated with activation of DNA strand breakage

Cell cycle instability is initiated *via* activation of DNA damage responses following genotoxic stress. An alkaline Comet assay was used to detect DNA strand breakages in BNIPDaCHM-treated MDA-MB-231 cells. The results indicate that

BNIPDaCHM (0.1-10 μM after 4 and 24 hours) induced a statistically significant dose dependent increase in DNA strand breakage, compared to endogenous levels (Fig. 5A and 5B). Also, MDA-MB-231 cells were significantly more sensitive to BNIPDaCHM treatment than the non-tumourigenic MCF-10A cell line: (251 *vs* 154 DNA strand breaks in MDA-MB-231 *vs* MCF-10A cells after 4 hours treatment with 0.1 μM BNIPDaCHM and, 335 *vs* 181 DNA strand breaks with 5 μM BNIPDaCHM ($P<0.01$) (data shown for BNIPDaCHM concentrations used in subsequent experiments). An increase in double strand breaks (DSBs) was shown by significantly increased levels of γ-H2AX-foci, compared to the control (Fig. 5C and 5D); with the maximum level of γ-H2AX-foci in MDA-MB-231 cells was observed at 24 hours with 5 μM BNIPDaCHM (Fig. 5C and 5D). This is the first time that DNA damage *via* DSBs produced after treatment with BNIPDaCHM has been qualitatively identified *in vitro*.

γ-H2AX-foci expression was also used to assess if the effect of BNIPDaCHM treatment on DNA damage was transient. MDA-MB-231 cells were washed free of BNIPDaCHM and allowed to recover in fresh medium for 2 hours. Results showed a persistence of BNIPDaCHM-induced DNA DSBs compared to the control and similar findings were observed when DNA repair of H_2O_2-induced DNA strand breakage was quantified after 4 and 24 hours pre-treatment with BNIPDaCHM. Cells were unable to effectively repair H_2O_2-induced DNA strand breaks; with MDA-MB-231 cell repair mechanisms significantly delayed (Fig. 5E and 5F).

Induction of DNA damage, particularly DSBs, may involve tumour protein p53, which can induce cell cycle instability *via* transcriptional targets, such as, $p21^{Waf/Cip1}$. Gene expression analysis, *via* RT-PCR, of MDA-MB-231 cells treated with BNIPDaCHM for up to 4 hours revealed no alteration in p53 mRNA levels (Fig.

6A). However, a significant time dependent increase (25%) in $p21^{Waf/Cip1}$ mRNA levels was observed (Fig. 6B), suggesting that BNIPDaCHM treatment modulates DNA damage independently from p53.

4. Discussion

In this study, the mechanism of action of a novel bisnaphthalimidopropyl derivative, BNIPDaCHM, was investigated in one of the most commonly studied TNBC cell lines. MDA-MB-231 cells are a highly aggressive and invasive TNBC cell line which provides an ideal *in vitro* model to determine the potential use of BNIPDaCHM in the treatment of TNBC. TNBC disease models, such as, MDA-MB-231, MDA-MB-468, HCC1806, BT-549, Cal-51 cells have recently been used to determine the potential anti-cancer effects of halogenated pyrrolo[3,2-*d*]pyrimidines [29], 6,7-bis(hydroymethyl)-1*H*,3*H*-pyrrolo[1,2-c]thiazoles [30] or celastrol [31], poly(ADP-ribose) polymerase inhibitors [32], and also, propolis and its derivative caffeic acid phenethyl ester [33], to name a few.

This present study delineates the underlying mechanisms utilised by BNIPDaCHM. A significant decrease in cell viability in a concentration ($\geq$ 5 μM) and time ($\geq$ 24 hours) dependent manner were observed. The mechanism of growth inhibition observed after treatment with BNIPDaCHM involved significant DNA strand breaks; identified for the first time to be DSBs observed by the increased expression of γ-H2AX-foci: a specific and sensitive surrogate marker for DNA DSBs [34]. Moreover, treatment with non-genotoxic concentrations of BNIPDaCHM induced a significant decrease in the repair of DNA strand breaks induced by H_2O_2. Also, an increase in the sub-G1 cell population (i.e., DNA fragmentation) corroborated the visual changes to cell morphology and plasma membrane integrity/permeability observed with BNIPDaCHM which indicated cell death.. Thus, interestingly, BNIPDaCHM's time-dependent influence on DNA repair capacity has identified a novel mechanism of action for bisnaphthalimide derivatives in a TNBC cell line. Also, BNIPDaCHM treatment resulted in p53-independent expression of

$p21^{Waf1/Cip1}$ suggesting that the requirement of p53 activation was not essential for BNIPDaCHM cytotoxicity: significant treatment effects were observed in a cell line with mutant p53. This study has shown the specific mechanism of action utilised by a bisnaphthalimidopropyl derivative in MDA-MB-231 cells and, has therefore identified a potentially novel anti-cancer agent.

Induction of cell cycle instability occurs in response to various stresses, including DNA damage. It is well known that cells have an inherent ability to effectively remove and repair DNA damage but, if repair mechanisms in the cell are faulty or the cell is overwhelmed by damage, chances are that the cells will be unable to repair and the damaged cell will be removed by apoptosis. DNA DSB damage directly induced by BNIPDaCHM is the key component in the toxicity of BNIPDaCHM in MDA-MB-231 cells. This mechanism is also consistent with other novel naphthalimide [35] and bisnaphthalimide derivatives [16, 24].

Finally, previous research has focused on the vital role of p53 in the mechanism of novel naphthalimide derivatives in p53 wild-type cancer cells [36, 37]; however, to the author's best knowledge, no research is available to reveal the mechanism(s) of the anti-cancer effect of bisnaphthalimide derivatives, particularly in mutant p53 cancer cells. Common in tumours is the generation of non-functional phenotypes which occur by mutations in p53 [38] and, although the frequency of mutations is lower in breast tumours than in other tumour types, mutant status can be positively associated with disease aggressiveness and overall tumour cell survival [39, 40]. It is therefore important that anti-cancer drugs are developed that can specifically target cells independent of their p53 status. This effect was observed for BNIPDaCHM in MDA-MB-231 breast cancer cells, which have a mutant, non-functional form of p53; with significant cytotoxicity and cell death. It has also been

shown that, in response to DNA damage, MDA-MB-231 cells undergo increased p53-independent expression of $p21^{Waf1/Cip1}$ and altered DNA repair capacity. The elevation of $p21^{Waf1/Cip1}$ expression through p53-independent pathways is not a new concept as it has been described in various cell types [41, 42]. However, this proposed mechanism of action is novel for a bisnaphthalimide derivative and thus, offers a significant therapeutic option for tumours with mutant p53, which is the direct cause of resistance to chemotherapy.

In conclusion, BNIPDaCHM treatment in MDA-MB-231 cells induced a significant induction of DNA DSBs and inhibition of DNA repair at non-genotoxic concentrations *via* p53-independent expression of $p21^{Waf1/Cip1}$ leading to cell death and the latter may be a consequence of a novel interaction between BNIPDaCHM and MDA-MB-231 cells. Further studies are warranted to provide a comprehensive assessment into the mechanism(s) of action of BNIPDaCHM and, the future design and development of bisnaphthalimide-based therapeutics, in different TNBC cell lines, to challenge the current treatment options available to breast cancers that are resistant to conventional chemotherapy.

Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

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GAB conducted the cytotoxicity, cell cycle, apoptosis (AO/EtBr staining and annexin V/FITC flow cytometry), DNA strand breakage and repair experiments, and RT-PCR experiments. GAB also collected and analysed the data and, wrote the manuscript. MG conducted the fluorescence microscopy of γH2AX along with IK and SI, collected and analysed the data. GAB, GB, MG and PK designed the study and all authors read and approved the submitted version of the manuscript.

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Figure Legends

Fig. 1. Structure of bisnaphthalimidopropyl diaminocyclohexylmethane (BNIPDaCHM).

Fig. 2. BNIPDaCHM reduced MDA-MB-231 cell viability. Dose-response curves. Cytotoxicity was determined by MTT assay. Results were obtained after treating MDA-MB-231 cells with different concentrations (0-40 µM) of BNIPDaCHM over 72 hours. Data are mean±SEM of 3 independent experiments (n=3). *P<0.05, **P<0.01 *vs.* DMSO-treated cells.

Fig. 3. BNIPDaCHM induced cell cycle instability. **(A)** Histograms of cell cycle distribution of MDA-MB-231 cells treated with BNIPDaCHM (5 µM) or etoposide (10 µM), with DMSO (0.025% v/v) as the solvent control, after 24 hours. Line markers (left to right) indicate the approximate cell cycle phases: sub-G1, G1, S and G2/M. Histograms are representative of 4 independent experiments (n=4). **(B)** Quantification of cell cycle profiles determined by flow cytometry *via* PI staining after 24 hours treatment with BNIPDaCHM (5 µM) or etoposide (10 µM), with DMSO (0.025% v/v) as the solvent control. 10,000 single events were recorded and the percentage of the cell population in sub-G1, G1, S and G2/M were calculated from histograms of linear FL-2 plots in the gated region. Data are presented as mean±SEM of 4 independent experiments, conducted in duplicate (n=4). *P<0.05, **P<0.01, ***P<0.001 *vs.* DMSO-treated cells.

Fig. 4. BNIPDaCHM induced changes in plasma membrane integrity (**A)** Changes in cell morphology determined by AO/EtBr staining after 0.5, 6 and 24 hours treatment

with BNIPDaCHM (5 µM). Images are representative of 3 independent experiments, conducted in duplicate (n=3). Arrows indicate viable cells (white arrow); early apoptotic cells (yellow arrow) and late apoptotic cells (red arrow). Magnification x200. **(B)** Histograms of apoptotic (1-4) and (**C**) necrotic (1-4) distribution of MDA-MB-231 cells. MDA-MB-231 cells (untreated: 1) were treated for 0.5 hours with DMSO (0.025% v/v) (2), 5 µM BNIPDaCHM (3) and 3% (v/v) formaldehyde (4) and stained with annexin V-FITC/7-AAD before flow cytometric analysis. Histograms are representative of 3 independent experiments (n=3). (**D**) Quantification of phosphatidylserine (PS) exposure and membrane integrity determined by flow cytometry following annexin V-FITC staining and 7-AAD labelling after 0.5, 4 and 6 hours treatment with BNIPDaCHM (5 µM). 10,000 single events were recorded. The percentage of cells labelled with annexin V but, not 7-AAD, were denoted % annexin positive whilst cells labelled with 7-AAD were denoted as %7-AAD positive. Data are mean±SEM of 3 independent experiments, conducted in duplicate (n=3).

Fig. 5. (A) Representative images of BNIPDaCHM-induced DNA damage (strand breaks) in MDA-MB-231 cells determined by Comet assay under alkaline conditions after 24 hours. **(B)** Quantification of DNA damage (strand breaks) was obtained after treating MDA-MB-231 cells with BNIPDaCHM (0-10 µM) for 4 and 24 hours. Camptothecin (10 µM; 2 hours, at 37 °C) and H_2O_2 (200 µM; 5 minutes, on ice) were used as positive controls. Data are mean±SEM of 2 independent experiments, conducted with 8 replicates (n=2). *P<0.05, **P<0.01 *vs.* DMSO-treated cells. **(C)** Representative images of γ-H_2AX-foci expression in MDA-MB-231 cells. MDA-MB-231 cells were treated with BNIPDaCHM (0.1 or 5 µM) for 4 and 24 hours, at 37 °C, and with Camptothecin (10 µM; 2 hours at 37 °C) + 2 hr recovery before detection.

Staining of γ-H_2AX-foci (Green as indicated by white arrows) and DNA (Blue) were detected using an γ-H2AX monoclonal antibody and DAPI, respectively. Images are representative of 3 independent experiments, conducted in duplicate (n=3). Magnification x400.**(D)** For quantification, γ-H2AX stained nuclei and DAPI stained nuclei in treated MDA-MB-231 cells were taken and analysed using QCapture Pro 6.0. γ-H2AX stained nuclei/DAPI stained nuclei ratio was calculated and expressed as a percentage. Data are mean±SEM of 3 independent experiments, conducted in duplicate (n=3). *P<0.05, **P<0.01 vs. DMSO-treated cells. Repair of DNA strand breaks in MDA-MB-231 cells. Cells were pre-treated in the presence (□) or absence (●) of non toxic BNIPDaCHM concentrations of 0.1 µM for 4 hours **(E)** or 1 µM for 24 hours **(F)**. Cells were incubated with H_2O_2 (200 µM, for 5 minutes on ice) and allowed to repair for up to 24 hours. At intervals during the 24 incubation, DNA strand breaks were analysed immediately (0 hours) or, after 2, 4, 8 and 24 hours, by Comet assay. UT represents cells which were not incubated with H_2O_2. Data are mean±SEM of 2 independent experiments, conducted with 8 replicates (n=2). *P<0.05, **P<0.01, ***P<0.001 *vs.* absence of BNIPDaCHM at each corresponding time point.

Fig. 6. BNIPDaCHM increased $p21^{Waf/Cip1}$ gene expression. Expression levels of p53 **(A)** and $p21^{Waf/Cip1}$ genes **(B)** in BNIPDaCHM-treated MBA-MB-231 cells by RT-PCR. MDA-MB-231 cells were treated with BNIPDaCHM (5 µM) for 1 or 4 hours. Total RNA was extracted and RT-PCR conducted with p53 and $p21^{Waf/Cip1}$ specific primers and β-actin as an internal control. Data are mean±SEM of 3 independent experiments, conducted in duplicate (n=3). **P<0.01 *vs.* DMSO-treated cells.

Table Legends

Table 1. Primers used in semi-quantitative polymerase chain reaction studies

Table 2. Cytotoxicity of BNIPDaCHM in MDA-MB-231 cells

Fig. 1.

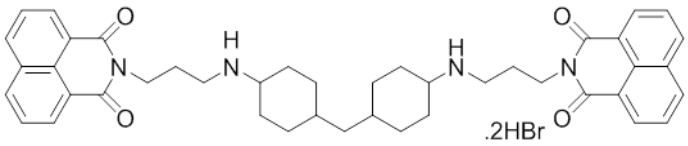


Fig. 2.

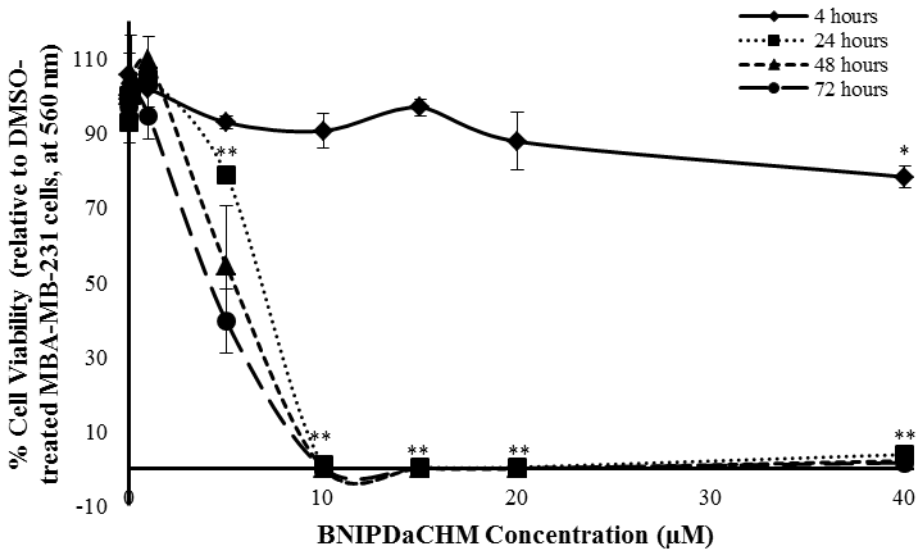

4 hours
24 hours
48 hours
72 hours
% Cell Viability (relative to DMSO-treated MBA-MB-231 cells, at 560 nm)
110
90
70
50
30
10
-10
0
10
20
30
40
BNIPDaCHM Concentration (µM)
*
**
**
**
**
**

Fig. 3.

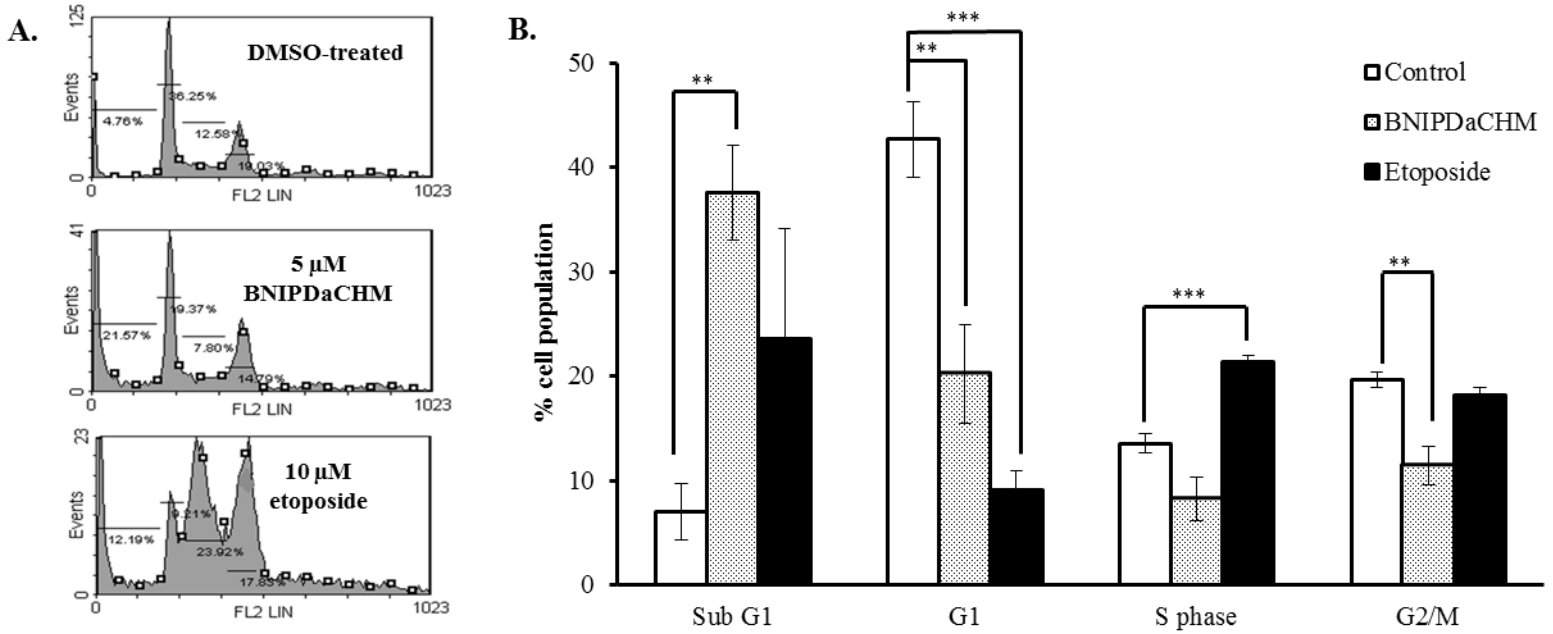
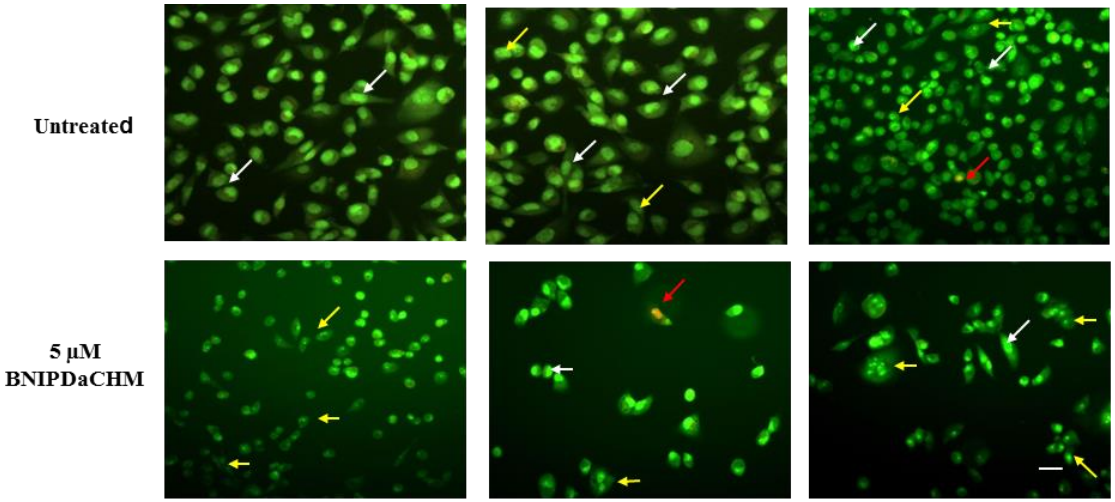
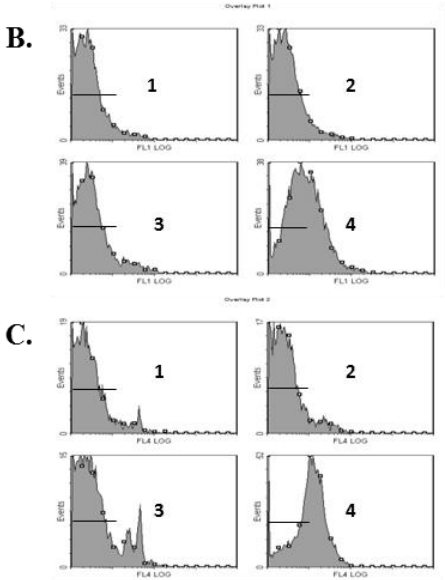

A.
DMSO-treated
36.25%
4.76%
12.58%
19.03%
Events
FL2 LIN
0
1023
125
5 µM
BNIPDaCHM
19.37%
21.57%
7.80%
14.79%
41
10 µM
etoposide
9.21%
12.19%
23.92%
17.85%
23
B.
% cell population
50
40
30
20
10
0
**
**
***
***
**
Control
BNIPDaCHM
Etoposide
Sub G1
G1
S phase
G2/M

Fig. 4.


Untreated
5 µM
BNIPDaCHM


B.
C.
1
2
3
4
FL1 LOG
FL4 LOG
Events

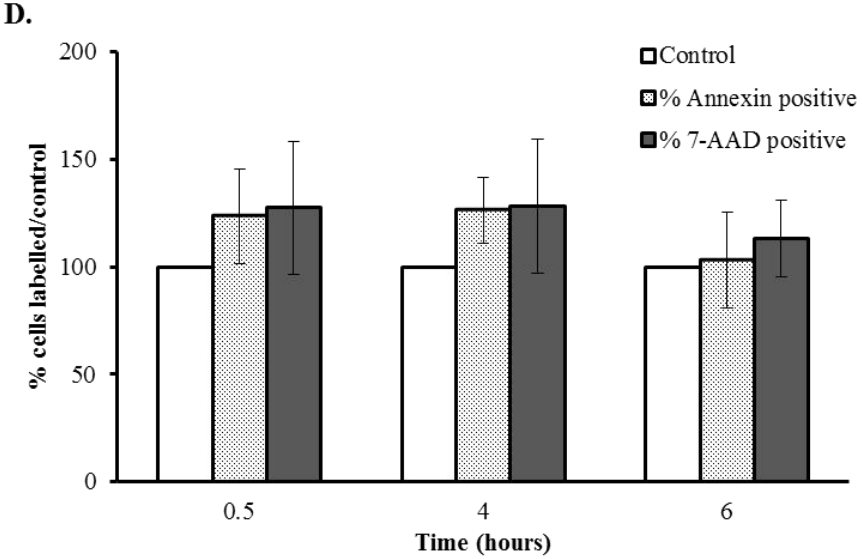

D.
% cells labelled/control
200
150
100
50
0
0.5
4
6
Time (hours)
Control
% Annexin positive
% 7-AAD positive

Fig. 5.

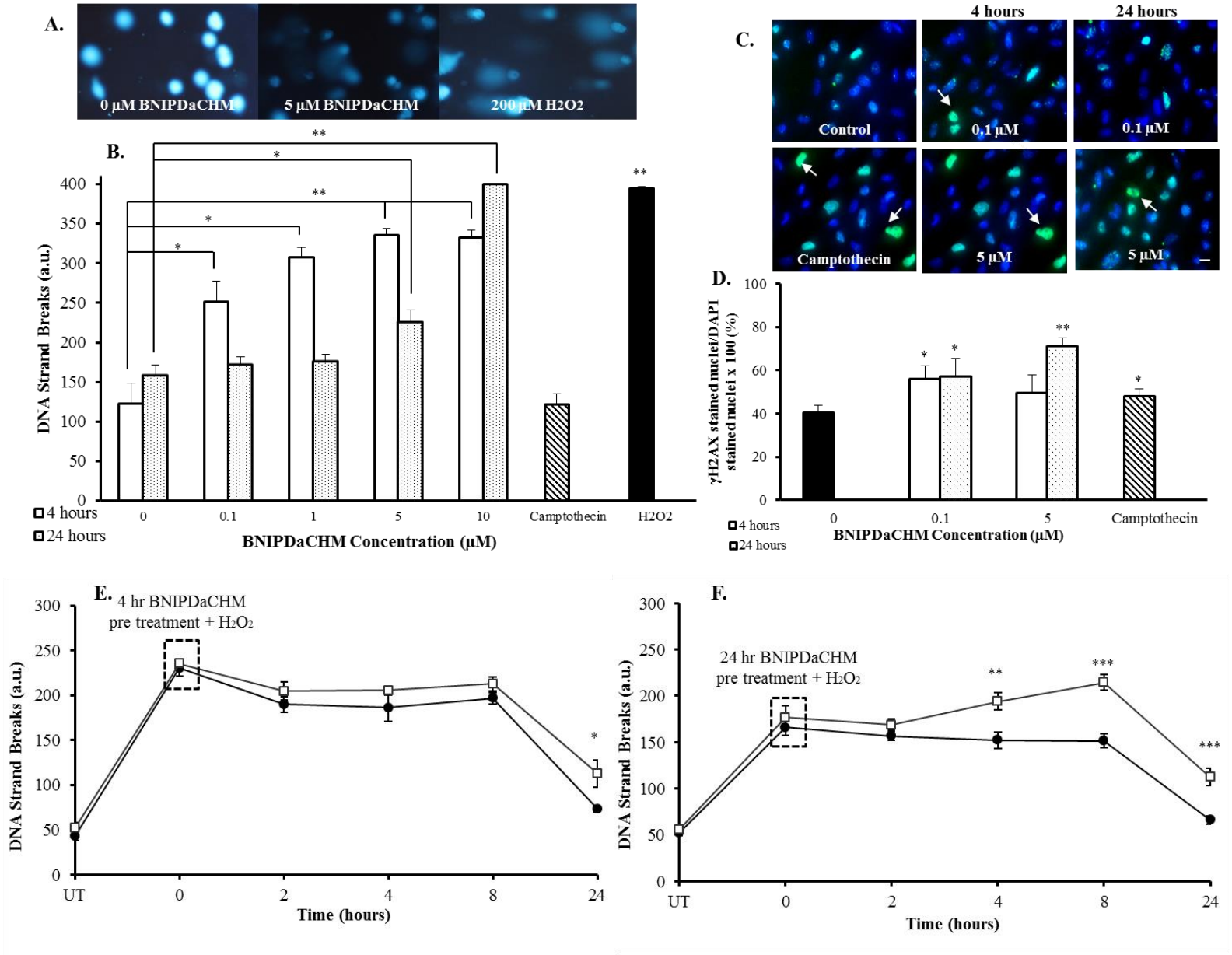

A.
0 µM BNIPDaCHM
5 µM BNIPDaCHM
200 µM H2O2
B.
DNA Strand Breaks (a.u.)
4 hours
24 hours
0
0.1
1
5
10
Camptothecin
H2O2
BNIPDaCHM Concentration (µM)
C.
4 hours
24 hours
Control
0.1 µM
0.1 µM
Camptothecin
5 µM
5 µM
D.
γH2AX stained nuclei/DAPI stained nuclei x 100 (%)
4 hours
24 hours
0
0.1
5
Camptothecin
BNIPDaCHM Concentration (µM)
E.
4 hr BNIPDaCHM pre treatment + H2O2
DNA Strand Breaks (a.u.)
UT
0
2
4
8
24
Time (hours)
F.
24 hr BNIPDaCHM pre treatment + H2O2
DNA Strand Breaks (a.u.)
UT
0
2
4
8
24
Time (hours)

Fig. 6.

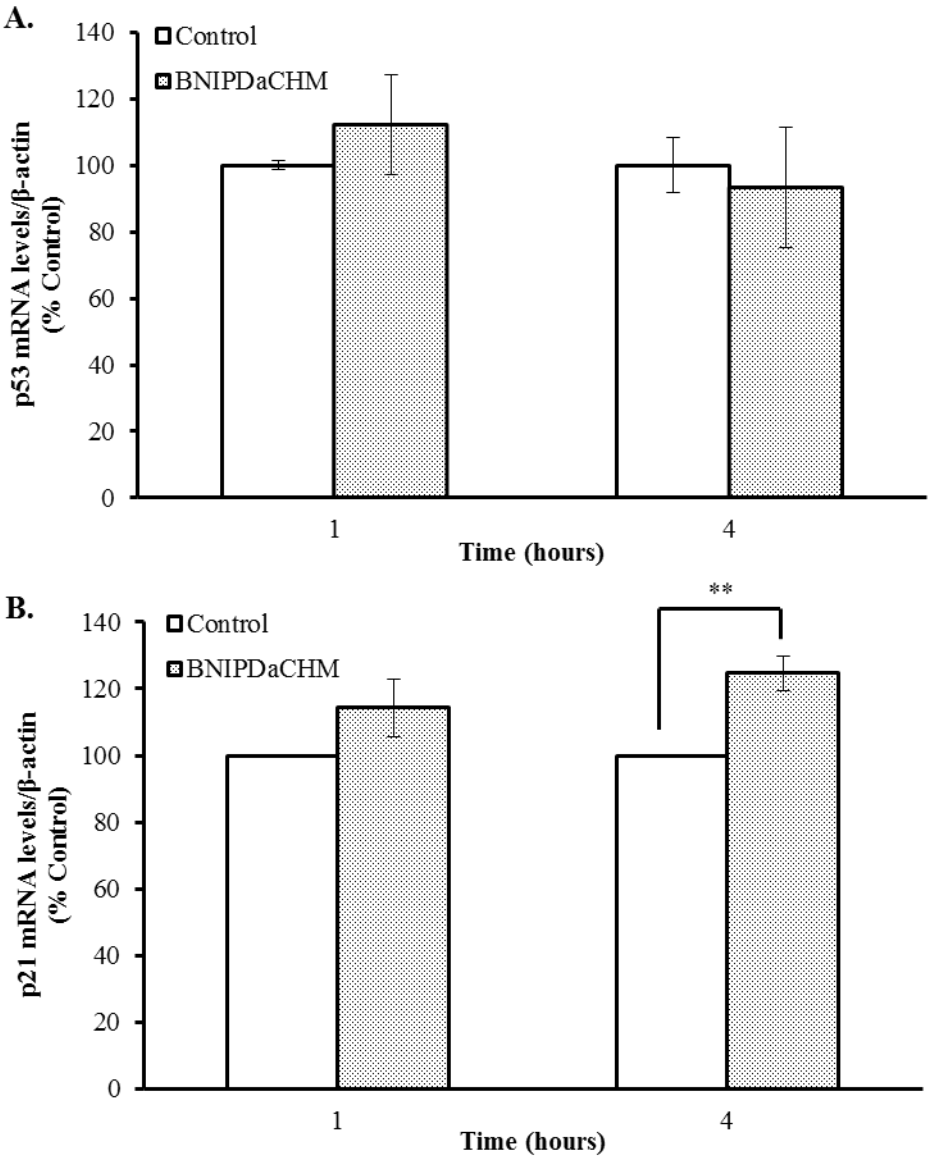

A.
Control
BNIPDaCHM
p53 mRNA levels/β-actin (% Control)
140
120
100
80
60
40
20
0
1
4
Time (hours)
B.
Control
BNIPDaCHM
**
p21 mRNA levels/β-actin (% Control)
140
120
100
80
60
40
20
0
1
4
Time (hours)

Table 1

Primer	Sequence	Product size (bp)
p53 F	CCAGGGCAGCTACGGTTTC	205
p53 R	CTCCGTCATGTGCTGTGACTG	
p21 $^{Waf1/Cip1}$ F	CCTGTCACTGTCTTGTACCCT	130
p21 $^{Waf1/Cip1}$ R	GCGTTTGGAGTGGTAGAAATCT	
β-actin F	CATGTACGTTGCTATCCAGGC	250
β-actin R	CTCCTTAATGTCACGCACGAT	

Table 2

Cell Line	IC50[1] (µM)			
	4 hours	**24 hours**	**48 hours**	**72 hours**
MDA-MB-231	> 40	6.8±0.1	5.2±0.6	4.6±0.9

[1]IC_{50} is the concentration required to inhibit cell growth by 50% of DMSO-treated cells (Dance *et al.* 2005). Cytotoxicity was determined by MTT assay. The results were obtained after treating MDA-MB-231 cells with different concentrations (0.01-40 µM) of BNIPDaCHM for over 72 hours at 37 °C. Data are mean±SEM of 3 independent experiments (n=3).