

In Vitro and *In Vivo* Anticancer Activity of a Novel Nano-sized Formulation Based on Self-assembling Polymers Against Pancreatic Cancer

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Received: 8 July 2010 / Accepted: 30 August 2010 / Published online: 25 September 2010
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ABSTRACT

Purpose To evaluate the *in vitro* and *in vivo* pancreatic anticancer activity of a nano-sized formulation based on novel polyallylamine grafted with 5% mole cholesteryl pendant groups (CH₅-PAA).

Methods Insoluble novel anticancer drug, Bisnaphthalimido-propyldiaminooctane (BNIPDaooct), was loaded into CH₅-PAA polymeric self-assemblies by probe sonication. Hydrodynamic diameters and polydispersity index measurements were determined by photon correlation spectroscopy. The *in vitro*

cytotoxicity evaluation of the formulation was carried out by the sulforhodamine B dye assay with human pancreatic adenocarcinoma BxPC-3 cells, while for the *in vivo* study, Xenograft mice were used. *In vitro* apoptotic cell death from the drug formulation was confirmed by flow cytometric analysis.

Results The aqueous polymer-drug formulation had a mean hydrodynamic size of 183 nm. The drug aqueous solubility was increased from negligible concentration to 0.3 mg mL⁻¹. CH₅-PAA polymer alone did not exhibit cytotoxicity, but the new polymer-drug formulation showed potent *in vitro* and *in vivo* anticancer activity. The mode of cell death in the *in vitro* study was confirmed to be apoptotic. The *in vivo* results revealed that the CH₅-PAA alone did not have any anti-proliferative effect, but the CH₅-PAA-drug formulation exhibited similar tumour reduction efficacy as the commercial drug, gemcitabine.

Conclusions The proposed formulation shows potential as pancreatic cancer therapeutics.

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KEY WORDS amphiphilic polyallylamine · apoptosis · Bisnaphthalimidopropyl-diaminooctane · BxPC-3 cells · nanoparticles · pancreatic cancer · self-assembling polymers

INTRODUCTION

Ductal adenocarcinoma of the pancreas is the fourth causative death from malignant disease in Western countries (1). Because of its aggressiveness, it usually leads to death. Pancreatic resection currently remains the only chance to cure patients, with a 5-year overall survival rate between 7% and 34% compared to a median survival of 3–11 months for unresected cancer patients (2–4). Despite the application of a new combination of chemotherapy and radiation therapy, prognosis remains very poor (5). Gemcita-

bine is currently the drug of choice for treatment and has a response rate of 23.8% in pancreatic cancer patients (6). However, the relatively low response of gemcitabine means that there is still an urgent need for new and more efficient therapies. One group of compounds which has shown promise as potential anticancer agents is the Bisnaphthalimidopropyl (BNIP) diaminoalkylamines. In our laboratory, BNIP derivatives, Bisnaphthalimidopropyl diamino-octane (BNIPDaoct), were designed and synthesised to exhibit good *in vitro* cytotoxicity against colon, breast and leukaemia cells (Fig. 1). Although cell death in those cell lines was confirmed to be apoptotic, the precise mode of action of those compounds is yet to be determined, although DNA damage is implicated (7–10). The lack of aqueous solubility associated with this group of compounds has made *in vitro* and *in vivo* testing extremely difficult (11). Many approaches have been attempted to address this issue. Chemical modifications of the bisnaphthalimides both at the naphthalimido rings and the linker alkyl chains has been attempted but with limited success (11,12).

Since BNIPDaoct showed negligible aqueous solubility, harsh solvents such as DMSO have to be used to get the drug into solution (7). Recently, Thompson and colleagues reported the fabrication of novel comb-shaped polymers based on the hydrophobic and hydrophilic modification of a water-soluble polymer backbone, poly(allylamine) (PAA) (13). The polymer forms nano self-assemblies upon the aggregation of the hydrophobic pendant groups in aqueous media and has shown to encapsulate hydrophobic probes (13). To date, the use of self-assembling polymers for the delivery of hydrophobic anticancer agents has been extensively studied (14). These nano-containers have shown to encapsulate poorly soluble anticancer drugs, such as paclitaxel, etoposide within their lipophilic core via hydrophobic interactions (15–17). During their *in vivo* journey, the nano-carriers protect the drugs from enzymatic degradation, resulting in an effective delivery to the target site (18). The advantages of using nano-carriers in chemotherapy include increased drug solubility, prolonged drug exposure time, tumour-selective drug delivery via enhanced permeability and retention effect, improved therapeutic efficacy, decreased side effects, and lower drug resistance (19–21). However, most of the self-assembling polymers consist of block copolymers which are formed mainly via copolymerisation of hydrophobic and hydrophilic monomers (18,19,21). In this report, we used a novel self-assembling

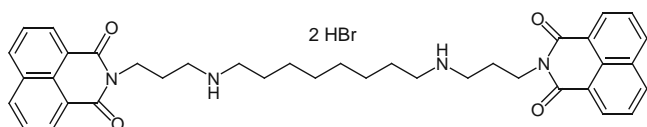


Fig. 1 Chemical structure of BNIPDaoct.

polymer based on modification of 15 kDa water-soluble polymer, polyallylamine (PAA) with cholesteryl moieties. There are limited studies on this type of polymer construct in drug delivery. To date, only a few reports using hydrophobically modified polyethylenimine (20), glycol chitosan (22), have shown promising potential in hydrophobic drug delivery. The cross-linked polyallylamine (PAA) has been used clinically as an oral phosphate binder (23); however, hydrophobically modified PAA as a delivery system for hydrophobic drugs has not been reported before. Furthermore, the use of modified PAA for biomedical application includes attachment of hydrophilic moiety (methyl glycolate) (24) or histidine to PAA for gene delivery (25), and the use of thiolated PAA as an intestinal permeation enhancer (26). None of the work thus far has attempted to attach a cholesteryl pendant group to PAA for hydrophobic drug delivery. Therefore, the novelty of this work lies in the *in vitro* and *in vivo* characterisation of a novel biomaterial for the delivery of an insoluble novel therapeutic agent. In this study, a formulation using the cationic PAA grafted with cholesteryl groups in 5% molar ratio (CH₅-PAA, Fig. 2) was applied to enhance the aqueous solubility of BNIPDaoct. The CH₅-PAA formulation incorporating BNIPDaoct was characterised, and the anticancer properties of such a formulation was studied both *in vitro* and *in vivo* using pancreatic BxPC-3 cancerous cells. The mechanism by which *in vitro* cell death occurs was explored using standard molecular approaches.

MATERIALS AND METHODS

Materials

Poly(allylamine) hydrochloride, cholesterol chloroformate, triethylamine, octane sulfonic acid, anhydrous sodium acetate, gemcitabine, carbonyl cyanide m-chlorophenylhydrazone and nonenzymatic cell-dissociation solution were from Sigma-Aldrich chemical company (UK). Thiopental was bought from

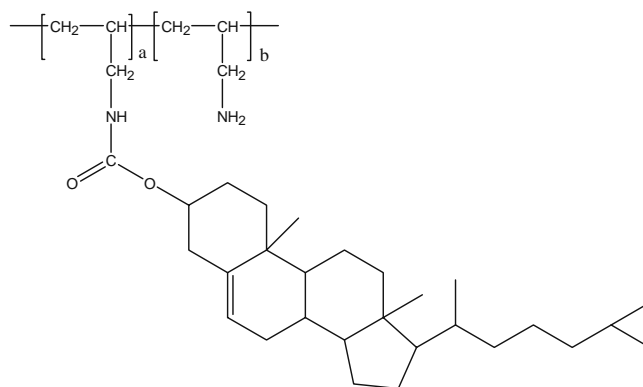


Fig. 2 Chemical structure of Cholesteryl-PAA (CH-PAA).

Sigma, St. Louis, MO. Visking tubing membranes were from Medicell International Ltd. (London, UK). GDX PVDF filters were purchased from Whatman, UK. DMSO, methanol and diethyl ether were from Fisher Scientific (UK). RPMI 1640 media, 10% heat-inactivated fetal bovine serum, 1% glutamine, L-glutamine, penicillin, streptomycin and amphotericin B were from BioWhittaker (Belgium). TCA (trichloroacetic acid) and 2% saponin were purchased from Fluka (Switzerland). Tetramethylrodamine methyl ester was purchased from Molecular probes. FITC-conjugated annexin detection kit was from R&D Systems.

Methods

Synthesis and Characterisation of 5% Cholesteryl-PAA

Synthesis of 5% mole modification of Cholesteryl-PAA (CH₅-PAA, Fig. 2) was carried out as described by Thompson and colleagues (30). Briefly, 15 kDa PAA-HCl (10 g, Sigma-Aldrich) salt was dissolved in doubly distilled water. Sodium hydroxide pellets were added slowly until a pH of 13 was achieved, and the mixture was stirred for 1 h. The polymer was exhaustively dialysed against water using 7000 Dalton membrane for 24 h with six water changes (at 2 h intervals for first 8 h). The solution was recovered from the dialysis tubing and freeze-dried on a 48 h cycle. The free amino PAA (2 g) was dissolved in 100 mL chloroform:methanol (1:1 v/v). Triethylamine (2 mL) was added, and the mixture was stirred for 0.5 h. Cholesteryl chloroformate (0.6678 g) was dissolved in 20 mL chloroform:methanol (1:1 v/v) and then added drop-wise to the polymer solution over 2 h at 37°C. The mixture was then left stirring for 24 h at 37°C.

After 24 h, the solvent was removed using a rotary film evaporator, and the residue washed with diethyl ether (3 × 100 mL). The dry residue was dissolved in doubly distilled water (50 mL), and the solution was exhaustively dialysed (molar weight cut-off = 7 kDa) against 5 L doubly distilled water for 24 h (6 water changes were made). The solution was freeze dried for 48 h, and the product was recovered as white cotton-like solid (2.2 g, 79% yield). The polymer was characterised by ¹HNMR (Bruker UltraShield 400 MHz) and elemental analysis (Strathclyde University, Glasgow, UK). BNIPDaoct was synthesised as previously reported (12).

Preparation of CH₅-PAA, BNIPDaoct Formulation

CH₅-PAA polymer solution (1 mg mL⁻¹) was prepared by dissolving the polymer in water followed by probe sonication (10 min). BNIPDaoct (1 mg mL⁻¹) was added to the CH₅-PAA solution and sonicated for a further 10 min to ensure maximum drug solubilisation had occurred. The solution was filtered using 0.45 µm syringe filters with prefilters to remove any excess undissolved drug (20).

Characterisation of Novel CH₅-PAA, BNIPDaoct Formulation

Solubilisation of BNIPDaoct

BNIPDaoct concentration in self-assemblies was analysed using RP Zorbax ODS 250 mm × 46 mm × 5 µm HPLC column (Hichrom, UK). The mobile phase consisted of 55:45 (v/v) buffer:acetonitrile, and the flow rate was 1 mL min⁻¹. The buffer for the mobile phase was made up of 0.432 g octane sulfonic acid and 1.64 g anhydrous sodium acetate made up to 200 mL with deionised water; the solution was subsequently pH adjusted to pH 4.5. The column eluent was monitored at 234 nm excitation and 394 nm emission, using a fluorescent detector (Shimadzu prominence UFLC, UK). The samples were diluted with the mobile phase, and 20 µL was injected onto the column; the resultant peak at 10 min was analysed. A calibration was carried out by dissolving BNIPDaoct in DMSO:water (50:50 v/v) (39–625 µg mL⁻¹), R² = 0.999. The drug loading efficiency was calculated using the following formula:

Drug loading efficiency

$$= \text{encapsulated drug analysed by HPLC} / \text{initial drug weight} \times 100$$

Sizing of Nano-aggregates

Hydrodynamic diameters and polydispersity index measurements were carried out on the CH₅-PAA alone and in polymer drug formulation using a photon correlation spectroscopy (PCS) (Zetasizer Nano-ZS, Malvern Instruments, UK). All measurements were conducted in triplicate at 25°C, and an average value was determined.

Cell Culture

Human pancreatic adenocarcinoma cell line BxPC-3 (ATCC) was maintained at 37°C in a humid atmosphere with 5% CO₂. The cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine, 2 mM L-glutamine, 100 U mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin and amphotericin B.

Cytotoxic Activity

Cell viability was evaluated using the sulforhodamine B dye assay (SRB; Sigma-Aldrich) (38). Cells were seeded on 96-well plates at a density of 2 × 10⁴ cells/well and incubated for 24 h at 37°C in 5% CO₂ atmosphere prior to the addition of different drugs and formulation. Cells were then treated with CH₅-PAA alone (0–30 µg/mL) and in CH₅-PAA-BNIPDaoct aqueous formulation (30 µg/mL polymer

and 0.04–10 μM drug); neat BNIPDaoct was dissolved in DMSO (50%) and gemcitabine in PBS, pH 7.4. The final concentration of DMSO used as control was 0.01%. After 48 h, the cells were fixed *in situ* by the addition of 50 μL of cold 50% (*w/v*) TCA (final concentration, 10% TCA) and incubated for 1 h at 4°C. Supernatants were discarded, and the plates were washed five times with water and air-dried. SRB solution (100 μL) at 0.4% (*w/v*) in 1% acetic acid was added to each well, and plates incubated for 10 min at room temperature. After staining, unbound dye was removed by five washes with 1% acetic acid, and then the plates were air dried. Bound SRB was subsequently solubilised with 200 μL of 10 mM Tris-base solution (pH 10.5) by agitating the plate on a shaker until the colour became homogeneous. SRB bound to the cellular protein content was determined by colorimetric measurement on an automated plate reader (515 nm). The IC_{50} values, defined as the drug concentration that inhibits 50% of growth compared to untreated cells, were then determined for each drug, polymer and formulation using the dose-dependent curves.

Detection of Apoptosis

Flow Cytometric Analysis of External Phosphatidylserine Exposure

Cells were seeded in 24-well plates at a density of 1×10^5 cells/well and allowed to adhere for 24 h followed by incubation with $\text{CH}_5\text{-PAA}$, $\text{CH}_5\text{-PAA-BNIPDaoct}$ formulation, BNIPDaoct or gemcitabine (1 or 10 μM) for 24 h. Cells were collected and labelled with FITC-conjugated annexin V for 15 min, at room temperature, in a Ca^{2+} -enriched binding buffer (apoptosis detection kit, R&D Systems). PI (propidium iodide) ($0.5 \mu\text{g mL}^{-1}$) was added to exclude the necrotic cells with disrupted plasma membrane permeability, and the cells were analyzed by flow cytometry. Staurosporine-exposed cells (1 μM) were used as a positive control. All data were analyzed using CellQuest software (BD Biosciences, USA).

Measurement of Mitochondrial Membrane Potential

Tetramethylrhodamine methyl ester (TMRE) is a cationic lipophilic dye that readily accumulates in active mitochondria. For the determination of mitochondrial membrane potential ($\Delta\Psi$), the cells were seeded in 24-well plates at a density of 1×10^5 cells/well and allowed to adhere for 24 h followed by incubation with $\text{CH}_5\text{-PAA}$, $\text{CH}_5\text{-PAA-BNIPDaoct}$ formulation, BNIPDaoct or gemcitabine (1 or 10 μM) for 24 h. Drug-treated or non-treated cells were washed in PBS and incubated with 200 nM of TMRE for 30 min at 37°C and 5% CO_2 and analyzed by flow

cytometry on the FL2-H channel. PI ($4 \mu\text{g mL}^{-1}$) was used before the last acquisition to exclude dead cells and recorded on FL3-H channel. As a positive control, cells already labelled with TMRE for 30 min were treated during 20 min at 37°C with 200 μM final concentration of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which depolarizes mitochondria by abolishing the proton gradient across the inner mitochondrial membrane (27).

Flow Cytometric Analysis of DNA Fragmentation

The DNA content of 1×10^5 cells/well incubated with 1 or 10 μM of $\text{CH}_5\text{-PAA}$, $\text{CH}_5\text{-PAA-BNIPDaoct}$ formulation, BNIPDaoct or gemcitabine during 24 h was determined by flow cytometry using PI ($0.5 \mu\text{g mL}^{-1}$), after the cells were permeabilized with 50 μL of 2% saponin solution in PBS (28). After permeabilization, drug-treated or non-treated cells were washed in PBS and then analyzed by flow cytometry on the FL3-H channel.

In Vivo Effect of $\text{CH}_5\text{-PAA-BNIPDaoct}$ Formulation on Xenograft Mice (Mice Implanted Subcutaneously with BxPC-3 Tumor Cells)

Female NMRI Nu/Nu mice, six weeks of age, (le Genest-St.-Isle, France) were kept in pathogen-free conditions (weight of mice was 24–30 g). All surgical procedures and animal care were carried out according to accreditation number 13416 given by the French ministry of agriculture. Human pancreatic cancer cell line BxPC-3 was cultured to 90% confluence in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U mL^{-1} penicillin, 100 $\mu\text{g mL}^{-1}$ streptomycin and amphotericin B. The cells were washed twice with cold PBS and harvested with nonenzymatic cell-dissociation solution for 10 min at 37°C. The cells were washed three times with PBS and kept on ice until injection. Mice were transiently anesthetized (<30 s) with a low dose (2.5 mg) of thiopental to place them in the restraining tube. The tumour cell suspension (1.8×10^6 cells in 100 μL of PBS) was injected subcutaneously (s.c.) in the right flank of each mouse. When the tumour became palpable (two weeks), measurements in two dimensions with vernier calipers were carried out once a week and volume tumours calculated according to the formula $(\pi/6) \times (a \times b^2)$, where *a* is the largest and *b* the smallest diameter of the tumour.

Drug treatments were performed twice per week for four weeks. Four groups of mice were studied: Group 1 mice ($n=13$) received intraperitoneal injections (i.p.) of 1 mg kg^{-1} $\text{CH}_5\text{-PAA}$, 0.3 mg kg^{-1} BNIPDaoct aqueous formulation in a volume of 100 μL ; Group 2 mice ($n=6$) received i.p. injections of 1 mg kg^{-1} $\text{CH}_5\text{-PAA}$ aqueous solution in a volume of 100 μL ; Group 3 mice ($n=11$)

received i.p. injections of 2.5 mg kg⁻¹ of gemcitabine dissolved in PBS.

The treatment efficacy was evaluated by the change in tumour volume during the treatment period. The measurement for the tumour volume was performed twice per week for four weeks. After five weeks of treatment, the mice were sacrificed. Differences between the means of unpaired samples were evaluated by the Mann Whitney test, and the results were considered statistically different when $p < 0.05$.

RESULTS

Synthesis and Characterisation of CH₅-PAA

The ¹HNMR confirmed the synthesis, and the elemental analysis showed the cholesteryl grafting value (4.7% mole) was in close agreement with the initial molar feed ratios (13). The proton assignments for CH₅-PAA are: $\delta_{0.75}$, $\delta_{0.9}$, $\delta_{1.0}$, $\delta_{1.1}$ = CH₃ (cholesteryl), $\delta_{1.1-2.1}$ = CH₂ (cholesteryl and PAA), $\delta_{2.3}$ = CH₂ (cholesteryl), $\delta_{2.4-3.2}$ = CH₂ (PAA), $\delta_{4.4}$ = CH-O (cholesteryl), $\delta_{5.4}$ = CH (cholesteryl).

Characterisation of Novel CH₅-PAA-BNIPDaoct Formulation

At 1 mg mL⁻¹, CH₅-PAA formed a clear colourless solution. The self-assemblies formed had a hydrodynamic radius of 167 nm (Table 1); the low polydispersity index indicated that the aggregates formed were mostly uniform in size. CH₅-PAA possessed a critical aggregation concentration (CAC) at 0.02 mg mL⁻¹; this was the lowest polymer concentration required for spontaneous self-assembly formation to occur (13). The CAC value was previously determined with the use of a hydrophobic methyl orange probe, whereby a hypsochromic shift was observed on encapsulation into the self-assemblies driven by non-covalent hydrophobic interactions (13).

The drug powder clumped together when it was mixed with water. Filtered BNIPDaoct aqueous solution showed negligible aqueous solubility as it was not detectable by HPLC. However, CH₅-PAA was capable of solubilising 0.3 mg mL⁻¹ of the BNIPDaoct at 1 mg mL⁻¹ polymer

concentration using 1:1 initial drug:polymer loading weight ratio. Filtration removed the excess undissolved drug in the formulation. Before filtration, the undissolved drug was clearly visible as yellowish solids clumped together at the top of the solution. Once filtered, the final solution was an optically clear yellowish solution indicating the encapsulation of the drug in the polymeric self-assemblies. The hydrodynamic radius of the polymeric self-assemblies increased from 167 nm to 183 nm in the presence of BNIPDaoct. The low PDI indicates uniform size population in the formulation (Table 1).

In Vitro Cytotoxic Activity

The human pancreatic cells (BxPC-3) were subjected to CH₅-PAA, CH₅-PAA-BNIPDaoct formulation, BNIPDaoct or gemcitabine treatments. The anti-proliferative effects were evaluated with final concentrations ranging from 0.04 to 10 μ M for 48 h, resulting in a dose-dependent inhibition of cell growth, quantified by SRB dye assay (Fig. 3). The polymer (CH₅-PAA) alone up to 30 μ g/mL and DMSO (0.01%) had no cytotoxicity on the pancreatic cancer cells, while CH₅-PAA-BNIPDaoct formulation and BNIPDaoct had a half maximal inhibitory concentration (IC₅₀) of 1.06 \pm 0.13 μ M and 1.11 \pm 0.12 μ M, respectively. The presence of polymer did not alter the cytotoxicity effect of BNIPDaoct. BxPC-3 cells exhibited a notably lower sensitivity to gemcitabine with an IC₅₀ value of 8.5 \pm 0.18 μ M, indicating that gemcitabine is less effective than BNIPDaoct.

In Vitro Apoptotic Analysis

Mediated CH₅-PAA-BNIPDaoct formulation and BNIPDaoct apoptosis were studied by the combination of a number of assays. Flow cytometry experiments were carried out at least three times and in duplicates (Figs. 4, 5, and 6). Although drug concentration of 5 μ M was attempted, similar results were obtained with 1 and 10 μ M drug concentrations (data not shown). With the exception of the DNA fragmentation assay, the cells analysed were gated for the live population. In general, the best results were obtained with compounds at 10 μ M concentration.

In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Substantial increase in phosphatidylserine exposure induced by CH₅-PAA-BNIPDaoct formulation (80%) and BNIPDaoct (90%) was observed using Annexin V/PI assays (Fig. 4) at 10 μ M drug concentration when compared with the positive control staurosporine (1 μ M). It is interesting to note that at 10 μ M drug concentration, both CH₅-PAA and gemcitabine showed similar levels of annexin V-positive

Table 1 Hydrodynamic Radius and Polydispersity Index of CH₅-PAA Polymer Solution and CH₅-PAA-BNIPDaoct Formulation Determined by Photon Correlation Spectroscopy

Polymer/Formulation	Size (nm)	Polydispersity index (PDI)
CH ₅ -PAA	167 \pm 3	0.190 \pm 0.100
CH ₅ -PAA-BNIPDaoct	183 \pm 2	0.167 \pm 0.001

All solutions was tested at 1 mg mL⁻¹ polymer concentration ($n = 3 \pm$ SD).

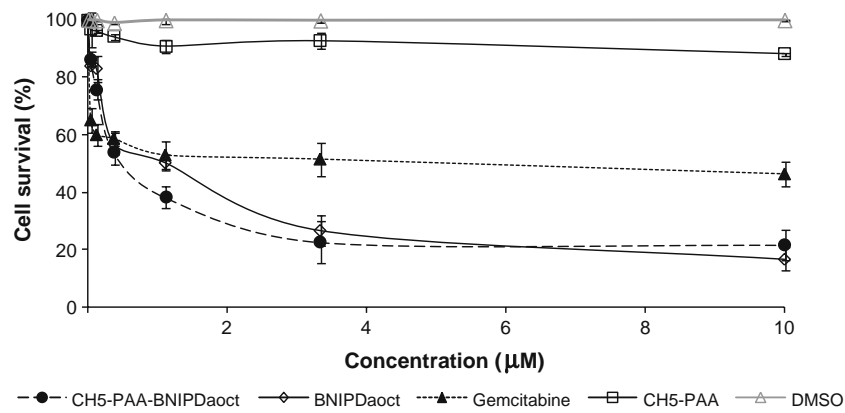


Fig. 3 Dose-dependent inhibition of BxPC-3 cell growth by CH₅-PAA-BNIPDaoct formulation, BNIPDaoct or gemcitabine (0–10 µM). The CH₅-PAA concentration used in all formulations was 30 µg/mL. CH₅-PAA alone was tested from 0 to 30 µg/mL and DMSO was at 0.01% concentration. Cells (2 × 10⁴/well) were seeded in 96-well plates and incubated overnight. Compounds were added at various concentrations, and cells were further incubated for 48 h. Cell proliferation was determined by SRB assay. The data is representative of three experiments carried out independently.

cells. No significant effect was detected with 1 µM drug concentration.

Mitochondrial damage as a result of depolarized Δψ_m, is often observed during the early apoptotic stages and may be a prerequisite for cytochrome c release (28). Therefore, experiments were carried out to determine whether drug-treated tumour cells induced a decrease in Δψ_m. The results shown in Fig. 5 demonstrated a significant increase in the percentage of cells exhibiting reduced Δψ_m upon drug treatment during 24 h with 1 or 10 µM of either CH₅-PAA-BNIPDaoct formulation or BNIPDaoct. The effect obtained was comparable to that observed with the positive

control treatment using CCCP and higher than that of the standard drug gemcitabine.

The above observations prompted us to investigate the changes occurring in the nuclear material of drug-treated cells by flow cytometry analysis after cell permeabilization and the labelling with PI. As shown in Fig. 6, both CH₅-PAA-BNIPDaoct formulation and BNIPDaoct at 10 µM concentrations and after 24 h exposure induced DNA fragmentation in BxPC-3 cells at a level significantly higher than that shown when using Staurosporine as a positive control. Furthermore those effects were five times the mean

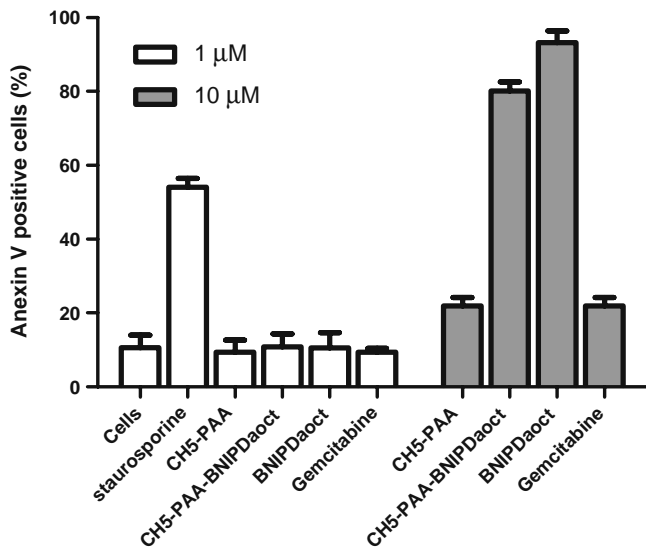


Fig. 4 Effect of CH₅-PAA, CH₅-PAA-BNIPDaoct formulation, BNIPDaoct or gemcitabine on phosphatidylserine exposure to BxPC-3 cells. Cells were treated during 24 h with either 1 or 10 µM drug. After PBS washing, cells were stained with annexin V-FITC and propidium iodide and analyzed by cytometry. Staurosporine (1 µM, 24 h) treatment was used as a positive control. Data are representative of three independent experiments. PI-positive cells were excluded.

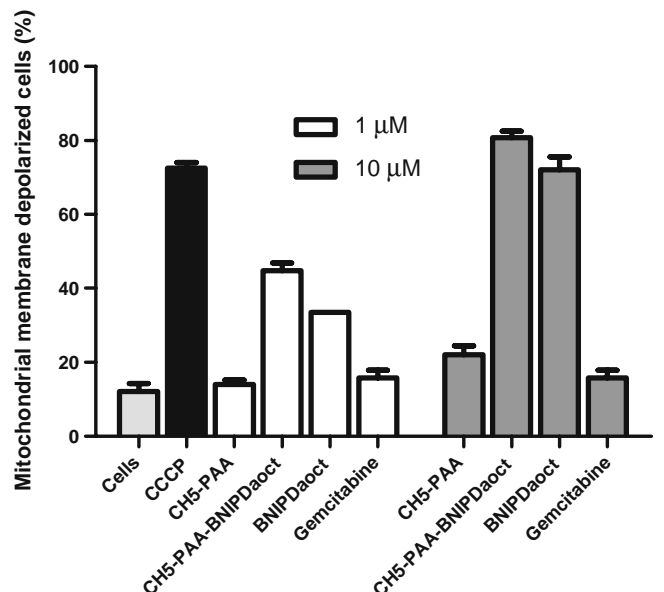


Fig. 5 Flow cytometry analysis of mitochondrial membrane depolarisation in BxPC-3 cells after 24 h treatment with 1 or 10 µM of either CH₅-PAA, CH₅-PAA-BNIPDaoct formulation, BNIPDaoct or gemcitabine. The percentage of cells exhibiting reduced Δψ_m was determined by flow cytometry using TMRE. CCCP was used as a positive control. Data are representative of three independent experiments. PI-positive cells were excluded.

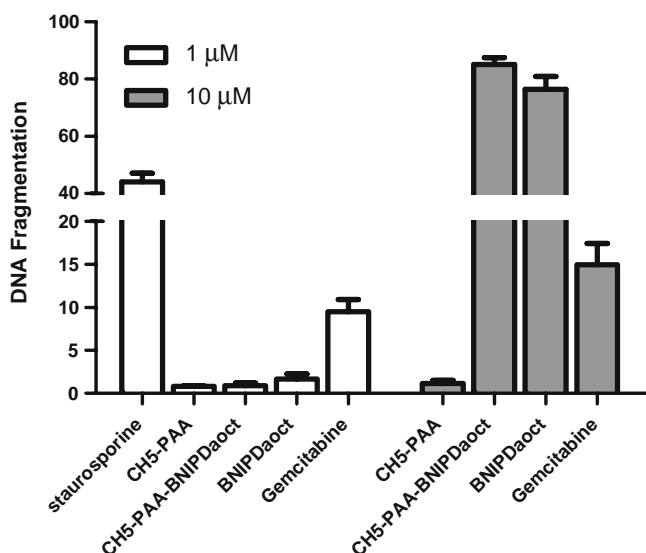


Fig. 6 Flow cytometry analysis of DNA fragmentation in tumor cells after incubation with CH₅-PAA, CH₅-PAA-BNIPDaoct formulation, BNIPDaoct or gemcitabine (1 or 10 μM). Cells with or without the drug treatment during 24 h were permeabilized with saponin, stained with propidium iodide and analyzed by cytometry. The positive control consisted of cells treated with 1 μM Staurosporine during 24 h. Data are representative of three independent experiments.

value observed when using 10 μM concentration of gemcitabine. Interestingly, in this experiment, polymer CH₅-PAA exhibited negligible DNA fragmentation, hence confirming the low toxicity of the polymer.

In Vivo Effect of CH₅-PAA-BNIPDaoct Formulation on Xenograft Tumour (BxPC-3 Cell Line Implanted)

To further analyze the anti-proliferative activity of the drug-formulation, the effect of BNIP derivative was assessed on mice which were implanted subcutaneously with BxPC-3 tumour cells. Figure 7 presents a scatter plot of mean tumour volumes after administration of drugs. All formulations were well tolerated with no gross toxicity reaction observed. Statistical analysis using the Mann Whitney test showed significant difference in tumour size between CH₅-PAA-BNIPDaoct formulation and CH₅-PAA-treated mice after 6, 9, 12, 16 days treatment (**p* values=0.028, 0.01, 0.003, 0.028 respectively). This indicates that CH₅-PAA itself did not exhibit any anti-proliferative activity. After the third injection, statistically significant difference in tumour size between CH₅-PAA-BNIPDaoct formulation and gemcitabine-treated mice was observed (*p*=0.006) (at day 6 after the beginning of IP treatment). Note that in this experiment the *in vivo* data showed the effectiveness of CH₅-PAA-BNIPDaoct formulation in reducing tumour growth in mice. The CH₅-PAA polymer alone was used as a negative control, i.e. tumour size keeps increasing with time, whereas

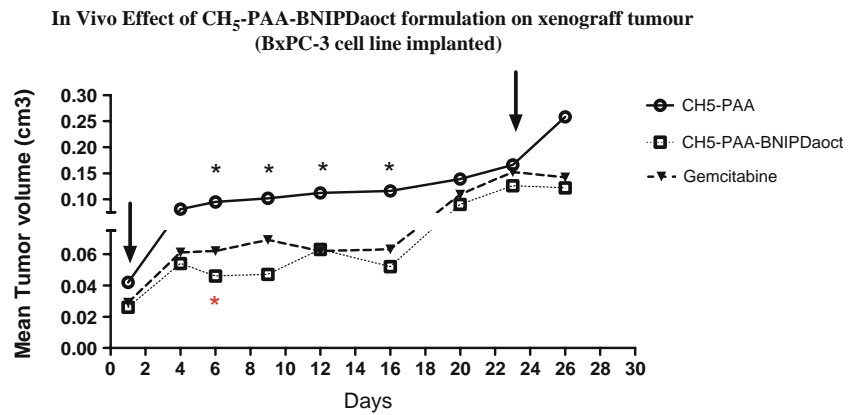
Gemcitabine, a known anticancer drug currently used in the clinic against pancreatic cancer, was used as a positive control. It is noteworthy to mention that there was no effect on tumor growth when mice were treated with PBS alone (data not shown).

At the end of the *in vivo* study termination, the mean tumour volume of each group was determined (Fig. 8). The Mann Whitney test was then applied to compare the values for each group. The group of mice treated with CH₅-PAA alone did not experience a reduction in tumour growth, while mice treated with both BNIPDaoct formulation (*p*=0.0159) and gemcitabine (*p*=0.0138) had a statistically significant decrease in tumour size compared to CH₅-PAA. This strongly supports that CH₅-PAA-BNIPDaoct formulation is at least as efficient as gemcitabine. Indeed, there is no statistical difference between the CH₅-PAA-BNIPDaoct formulation and gemcitabine (Fig. 8).

DISCUSSION

Most pancreatic cancers are not diagnosed until after they have metastasized. Standard treatments for advanced disease include radiotherapy and/or chemotherapy regimens. However, radiotherapy is often toxic, and the chemotherapy which includes drugs such as 5-fluorouracil (5-FU) and gemcitabine is either ineffective or effective for only a short duration. Although gemcitabine has been shown to provide an improvement in the life quality (29), high intrinsic resistance of pancreatic cancer to currently available agents might explain the failure of gemcitabine alone and gemcitabine-based combination chemotherapy to achieve great success (30–32). Therefore, new therapeutic strategies are urgently needed. The search for means to interfere with the tumour cell proliferation led to the identification of a large number of natural and synthetic compounds that could revert the cell morphology of various cancer cells to apparently normal phenotype (33). Interestingly, some of these molecules exhibited histone deacetylase inhibitory activity. For instance, pancreatic adenocarcinoma cell lines IMIM-PC-1, IMIM-PC2 and RWP-1 have been shown to be highly sensitive to the apoptosis-inducing effect of Trichostatin (TSA) and suberoylanilide hydroxamide (SAHA) (34). Moreover, it has been reported that TSA could synergize with gemcitabine (35) or proteasome inhibitor PS-341 to induce apoptosis of pancreatic tumor cell lines (36). We have recently reported that a number of class I, II and III deacetylase inhibitors, bisnaphthalimido-propyl (BNIPDaoct) could induce apoptosis of various human pancreatic cancer cell lines *in vitro* (38,39). The above observations provide a rationale to investigate further the anti-pancreatic tumour properties of BNIPDaoct. The bulky multi-ring structures and long alkyl chains of BNIPDaoct are

Fig. 7 Scatter plot of mean tumour volume in mice implanted with BxPC-3 cell line and treated with CH₅-PAA, CH₅-PAA-BNIPDaoct formulation, or gemcitabine. Treatment was started after 2 weeks of xenograft when tumour was palpable as described under Materials and Methods section. Error bars are SD. (**P* < 0.05, CH₅-PAA-BNIPDaoct formulation vs CH₅-PAA; ***P* < 0.05, CH₅-PAA, BNIPDaoct formulation vs gemcitabine). Arrows indicate the first and the last injection.



essential to preserve the activity of the drug. However, at the same time, this has resulted in undesirable physico-chemical properties of the drug. Negligible concentration of BNIPDaoct was obtained when the drug was dissolved in aqueous solution. As a result, *in vitro* assays were conducted using harsh solvents such as DMSO reported in our previous work (39). The poor aqueous solubility of the drug also restricts *in vivo* investigations, since a drug is commonly dissolved in aqueous media for *in vivo* parenteral administration.

Using the nano-carriers formed by amphiphilic polymer CH₅-PAA, an aqueous formulation of CH₅-PAA-BNIPDaoct was successfully prepared with a drug loading efficiency of 30%. Upon incorporation of the novel anticancer drug BNIPDaoct into the polymeric self-assembly, a slight increase in aggregate size was observed. The size increased from 167 nm (unloaded CH₅-PAA) to 183 nm in the presence of the drug (Table 1). This increase in

hydrodynamic radius can be attributed to expansion of the hydrophobic core in order to accommodate the BNIPDaoct molecules (40). The polydispersity index was slightly lower for the CH₅-PAA-BNIPDaoct formulations (0.167) than for the CH₅-PAA (0.190), indicating the drug-loaded aggregates were of a more uniform size distribution. The size of 178 nm is ideal, since it has been reported the size of <183 nm is important to ensure long circulation time *in vivo* (19)—the reason being, at this size, the nanoparticles are able to avoid the uptake of mononuclear phagocytic system (MPS) present in the liver and spleen. As a result, prolonged circulation will lead to accumulation at the tumour through the enhanced permeation and retention (EPR) effect (19).

When human pancreatic (BxPC-3) cells were exposed to the novel CH₅-PAA-BNIPDaoct formulation *in vitro*, they possessed a notably lower IC₅₀ than that of commercially available gemcitabine (8.50 μM), demonstrating that BxPC-3 cells are more sensitive to the cytotoxic effect of the polymer-drug formulation. The difference between the IC₅₀ of the free drug dissolved in DMSO and the CH₅-PAA-BNIPDaoct formulation was negligible. This indicated that the polymer did not enhance the cytotoxic effect of the drug on the cells, and this corresponds with the non-cytotoxic profile of CH₅-PAA at the concentrations tested. However, the formulation is still advantageous for *in vivo* administration as it is desirable to eliminate the use of harsh solvents which can cause toxic side effects to the patient. The non-volatile nature of the formulation will result in greater dose reproducibility and ease of administration. *In vitro* analysis of the CH₅-PAA-BNIPDaoct formulation and BNIPDaoct alone confirm that both caused cell death by apoptosis. Interestingly, the data demonstrated that CH₅-PAA had negligible toxicity toward the tumor cell line. Furthermore, the CH₅-BNIPDaoct formulation administered *in vivo* to nude tumour bearing mice was capable of reducing tumour growth rate when compared to CH₅-PAA alone.

Although the BNIPDaoct dose used in the formulation is eight-fold less than gemcitabine, the reduction in tumour growth was comparable to the current commercially

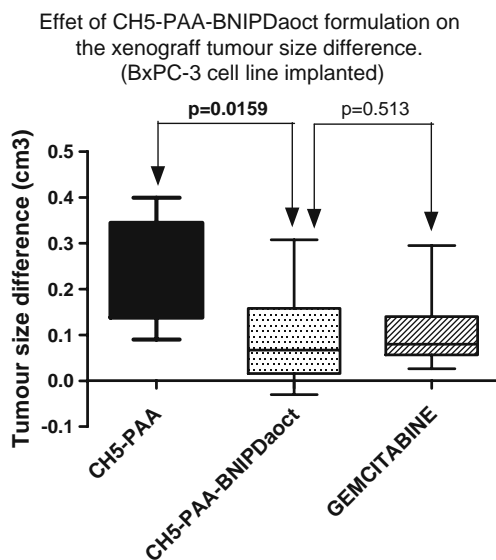


Fig. 8 The tumour size differences for each group were calculated using the following formula: tumour size at the end-tumour size at the start of treatment.

available drug gemcitabine for anticancer treatment. The reduction in tumour size was significant using the CH₅-PAA-BNIPDaoct formulation ($p < 0.05$) compared to the polymer alone, CH₅-PAA. It has been reported that glycol chitosan modified with sterol structure, cholanic acid, was able to entrap docetaxel with the particle size of 320 nm and exhibited higher antitumour efficacy in lung cancer cell-bearing mice than free docetaxel. The authors showed reduced *in vivo* anticancer drug toxicity compared to free docetaxel and attributed this effect to the ability of nano-sized particles to preferentially localized in tumour tissues (22). Chytil and colleagues demonstrated that N-(2-hydroxypropyl)methacrylamide (HPMA)-doxorubicin conjugate modified with cholesteryl pendant groups resulted in significant tumour regression with long-term survival in mice bearing EL-4T cell lymphoma compared to other alkyl chains substituents such as dodecyl and oleic acid (40). This indicates that the presence of cholesterol moieties is beneficial, although the exact mechanism is not known. In this study, comparison between free drug and the formulation was not possible due to the insolubility of the drug in aqueous solution for *in vivo* administration. Based on other similar work, it is hypothesized that EPR effect resulted in the antitumour efficacy observed in this study.

CONCLUSION

Pancreatic cancer remains an untreatable disease, which in most cases is uniformly lethal. Here we show that polyallylamine grafted with cholesteryl moieties (CH₅-PAA) was able to increase the water solubility of a practically insoluble anticancer agent, BNIPDaoct. The drug-loaded self-assembled aggregates had a mean diameter of 178 nm with a narrow size distribution. This formulation showed a greater cytotoxic effect *in vitro* on human pancreatic carcinoma cells than the leading drug gemcitabine in human pancreatic cancer cells, BxPC-3, while the polymer itself was non-cytotoxic. Although the BNIPDaoct dose was eight-fold less than gemcitabine, the formulation was able to reduce tumour growth in xenograft mice with results comparable to gemcitabine. The results of this study indicate that CH₅-PAA-BNIPDaoct has great potential as a suitable therapeutic alternative to gemcitabine for treatment of pancreatic cancer.

ACKNOWLEDGEMENTS

SCL is supported by a fellowship from FCT (SFRH/BPD/37880/2007). Clare Hoskins was funded by Robert Gordon University, Research Development Initiative scheme.

Conflict of Interest The authors do not have any conflicts of interest to report.

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