In Vitro and *in Vivo* Anticancer Activity of a Novel nano-sized Formulation based on self-assembling polymers against Pancreatic Cancer

Clare Hoskins[1], Mehdi Ouaissi[2,6], Sofia Costa Lima[3], Woei Ping Cheng[4], Inês Loureirio[3], Eric Mas[2], Dominique Lombardo[2], Anabela Cordeiro[3], Ali Ouaissi[3,5] and Paul Kong Thoo Lin[1]*.

[1]Robert Gordon University, School of Pharmacy and Life Sciences
St. Andrew Street, Aberdeen AB25 1HG, Scotland (UK)

[2]UMR-911 CRO2 INSERM, Faculté de Médecine-Timone, 27 Blv Jean MOULIN
13385 Marseille Cedex 05 - France

[3]IBMC – Instituto de Biologia Molecular e Celular
Universidade do Porto, Rua do Campo Alegre 823, 4150-180 Porto, Portugal

[4]University of Hertfordshire, School of Pharmacy
College Lane, Hatfield AL10 9AB, United Kingdom

[5]INSERM, CNRS, UMR 5235, Universite de Montpellier 2
Batiment 24-CC 107, Pl. Eugene Bataillon
34095 Montpellier Cedex 5, France

[6] Université Aix Marseille, Faculté de Médecine, Marseille, F-13000, France

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* To whom correspondence should be addressed

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 (CH_5-PAA).
Method. Insoluble novel anticancer drug, Bisnaphthalimidopropyldiaaminooctane (BNIPDaoct) was loaded into CH_5-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 the *in vivo* study, Xenograff mice were used. *In vitro* apoptotic death cell from the drug formulation was confirmed by flow cytometric analysis.
Results. The aqueous polymer-drug formulation had a mean hydrodynamic size of 183nm. The drug aqueous solubility was increased from negligible concentration to 0.3mgmL^{-1}. CH_5-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 CH_5-PAA alone did not have any anti-proliferative effect but the CH_5-PAA-drug formulation exhibited similar tumour reduction efficacy as the commercial drug, gemcitabine.
Conclusions. The proposed formulation shows potential as pancreatic cancer therapeutics.

INTRODUCTION

Ductal adenocarcinoma of the pancreas is the fourth causative death from malignant disease in the Western Countries (1). Because of its aggressiveness it usually leads to death sentence. Pancreatic resection currently remains the only chance to cure patients, with a 5-year overall survival rate between 7–34 % compared to a median survival of 3-11 months for unresected cancer patients. (2-4). Despite the application of new combination of chemotherapy and radiation therapy, prognosis remains very poor (5). Gemcitabine is currently the drug of choice for treatment with the 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, are the Bisnaphthalimidopropyl (BNIP) diaminoalkylamines. In our laboratory BNIP derivatives, Bisnaphthalimidopropyldiaminooctane (BNIPDaoct) had been 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 compound 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 had been attempted but with limited success (11, 12).

Figure 1

Figure 2

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, 20, 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 polymer based on modification of 15kDa water soluble polymer, polyallylamine (PAA) with cholesteryl moieties. There are limited studies of 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 include 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 on 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 (CH_5-PAA, Fig. 2) was applied to enhance the aqueous solubility of BNIPDaoct. The CH_5-PAA formulation incorporating BNIPDaoct was characterised and the anticancer properties of such formulation was studied in both *in vitro* and *in vivo* using pancreatic BxPC3 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 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-gluthamine, penicillin, streptomycin and amphotericine B were from BioWhitaker (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 (CH_5-PAA, Fig. 2) was carried out as described by Thompson and colleagues [30]. Briefly 15kDa 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 6 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 x 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 ^{1}HNMR (Bruker UltraShield 400 MHz) and elemental analysis (Strathclyde University, Glasgow, UK). BNIPDaoct was synthesised as previously reported (12).

Preparation of CH_5-PAA, BNIPDaoct formulation

CH_5-PAA polymer solution (1 $mgmL^{-1}$) was prepared by dissolving the polymer in water followed by probe sonication (10 min). BNIPDaoct (1 $mgmL^{-1}$) was added to the Ch_5-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.

Characterisation of novel CH_5-PAA, BNIPDaoct formulation

Solubilisation of BNIPDaoct

BNIPDaoct concentration in self-assemblies was analysed using RP Zorbax ODS 250 mm x 46 mm x 5 µm HPLC column (Hichrom, UK). The mobile phase consisted of 55:45 (v/v) buffer:acetonitrile and the flow rate was 1 $mLmin^{-1}$. 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, which 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 μgmL^{-1}), $R^2 = 0.999$.

Sizing of Nano-aggregates

Hydrodynamic diameters and polydispersity index measurements were carried out on the CH_5-PAA alone and 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% CO_2. The cells were grown in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 1% glutamine, 2 mM L-gluthamine, 100 Uml^{-1} penicillin, 100 μgmL^{-1} streptomycin and amphotericine B.

Cytotoxic Activity

Cell viability was evaluated using the sulforhodamine B dye assay (SRB; Sigma-Aldrich) [42]. Cells were seeded on 96-well plates at a density of 2 x 10^4 cells/well and incubated for 24 h at 37 °C in 5% CO_2 atmosphere prior to the addition of different drugs and formulation. Cells were then treated with CH_5-PAA alone (0-30 μg/mL), CH_5-PAA-BNIDaoct aqueous formulation (30 μg/mL polymer and 0.04-10 μM drug), neat BNIPDaoct was dissolved in DMSO and gemcitabine in PBS, pH 7.4. 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 mins 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 x 10^5 cells/well and allowed to adhere for 24 h followed by incubation with CH_5-PAA, CH_5-PAA-BNIDaoct 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 μgmL^{-1}) was added to exclude the necrotic cells with disrupted plasma membrane permeability, and the cells were analyzed by flow cytometry. Staurosporine-exposed cells (1μM) 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 x 10^5 cells/well and allowed to adhere for 24 h followed by incubation with CH_5-PAA, CH_5-PAA-BNIDaoct 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 μgmL^{-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 x 10^5 cells/well incubated with 1 or 10 µM of CH_5-PAA, CH_5-PAA-BNIDaoct formulation , BNIPDaoct or gemcitabine during 24 h was determined by flow cytometry using PI (0.5 μgmL^{-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 CH_5-PAA-BNIPDaoct formulation on Xenograff mice (Mice implanted subcutaneously with BxPC3 tumor cells)

Female NMRI Nu/Nu mice 6 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 04333 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 UmL^{-1} penicillin, 100 μgmL^{-1} streptomycin and amphotericine 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 3 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 ($1.8x10^6$ cells in 100 µL of PBS) was injected subcutaneously (s.c.) in the right flank of each mouse. When the tumour became palpable (2 weeks), measurements in 2 dimensions with vernier calipers were carried out once a week and volume tumours calculated according to the formula: (π/6) x (a x b^2), where a is the largest and b the smallest diameter of the tumour.

Drug treatments were performed twice per week during 4 weeks. Four groups of mice were studied: Group 1: Mice (n=13) received intraperitoneal injections (i.p.) of $1mgkg^{-1}$ CH_5-PAA, 0.3 $mgkg^{-1}$ BNIPDaoct aqueous formulation in a volume of 100 µL. Group 2: Mice (n=6) received i.p. injections of $1mgkg^{-1}$ CH_5-PAA aqueous solution in a volume of 100 µL. Group 3: A group of mice (n=11) received i.p. injections of 2.5 $mgkg^{-1}$ 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 during 4 weeks. After 5 weeks of treatment, the mice were sacrificed. Difference 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 CH_5-PAA

The ^{1}HNMR 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 CH_5-PAA are: $\delta_{0.75}$, $\delta_{0.9,}$ $\delta_{1.0,}$, $\delta_{1.1}$ = CH_3 (cholesteryl), $\delta_{1.1\text{-}2.1}$ = CH_2 (cholesteryl and PAA), $\delta_{2.3}$ = CH_2 (cholesteryl), $\delta_{2.4\text{-}3.2}$ = CH_2 (PAA), $\delta_{4.4}$ = CH-O (cholesteryl), $\delta_{5.4}$ = CH (cholesteryl).

Characterisation of novel CH_5-PAA-BNIPDaoct formulation

At 1 $mgmL^{-1}$, CH_5-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. CH_5-PAA possessed a critical aggregation concentration (CAC) at 0.02 $mgmL^{-1}$, 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).

Table 1

The drug solubilisation capacity of the PAA amphiphile was determined using HPLC analysis. CH_5-PAA was capable of solubilising 0.3 $mgmL^{-1}$ of the BNIPDaoct at 1 $mgmL^{-1}$ polymer concentration using 1:1 initial drug:polymer loading weight ratio. The yellowish drug powder clumped together when it was mixed with water. Filtered BNIPDaoct aqueous solution showed negligible aqueous solubility while in the presence of PAA polymeric self-assemblies, an optically clear and yellowish solution was formed. 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 (BxPC3) were subjected to CH_5-PAA, CH_5-PAA-BNIDaoct formulation, BNIPDaoct or gemcitabine treatments. The anti-proliferative effects were evaluated with final concentrations ranging from 0.04 to 10 μM for 48 h, resulting in a dose-dependent inhibition of cell growth, quantified by SRB dye assay (Fig. 3). The polymer (CH_5-PAA) alone up to 30 μmg/mL had no cytoxicity on the pancreatic cancer cells (supplementary data) while CH_5-PAA-BNIPDaoct formulation and BNIPDaoct had a half maximal inhibitory concentration (IC_{50}) of 1.06 ± 0.13 μM and 1.11 ± 0.12 μM, respectively. The presence of polymer did not alter the cytotoxicity effect of BNIPDaoct. BxPC3 cells exhibited a notably lower sensitivity to gemcitabine with an IC_{50} value of 8.5 ± 0.18 μM indicating that gemcitabine is less effective than BNIPDaoct.

Figure 3

In Vitro Apoptotic Analysis

Mediated CH_5-PAA-BNIDaoct formulation and BNIPDaoct apoptosis were studied by the combination of a number of assays. Flow cytometry experiments were carried out at least 3 times and in duplicates (Fig. 4-6). Although drug concentration of 5μM was attempted, similar results were obtained with 1 and 10 μM 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 μM 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 CH_5-PAA-BNIPDaoct formulation (80%) and BNIPDaoct (90%) were observed using Annexin V/PI assays (Fig. 4) at 10 μM drug concentration when compared with the positive control staurosporine (1 μM). It is interesting to note that at 10 μM drug concentration, both CH_5-PAA and gemcitabine showed similar levels of annexin V positive cells. No significant effect was detected with 1 μM drug concentration.

Figure 4

Mitochondrial damage as a result of depolarized Δψm, 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 Δψm. The results shown in Fig. 5 demonstrated a significant increase in the percentage of cells exhibiting reduced Δψm upon drug treatment during 24h with 1 or 10 μM of either CH_5-PAA-BNIDaoct 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.

Figure 5

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 Figure 6, both CH_5-PAA-BNIDaoct formulation and BNIPDaoct at 10 μM concentrations and after 24h exposure induced DNA fragmentation in BxPC3 cells at a level significantly higher than that shown when using Staurosporine as a positive control. Furthermore those effects were five times the mean value observed when using 10 μM concentration of gemcitabine. Interestingly in this experiment,

polymer CH_5-PAA exhibited negligible DNA fragmentation hence confirming the low toxicity of the polymer.

Figure 6

In Vivo Effect of CH_5-PAA-BNIPDaoct formulation on xenograff tumour (BxPC3 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 BxPC3 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 CH_5-PAA-BNIPDaoct formulation and CH_5-PAA treated mice after 6, 9, 12, 16 days treatment (*p values= 0.028, 0.01, 0.003, 0.028 respectively). This indicates that CH_5-PAA itself did not exhibit any anti-proliferative activity. After the 3rd injection, statistically significant difference in tumour size between CH_5-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 CH_5-PAA-BNIPDaoct formulation in reducing tumour growth in mice. The CH_5-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.

Figure 7

Figure 8

At the end of the *in vivo* study termination, the mean tumour volume of each group was determined. The Mann Whitney test was then applied to compare the values for each group. The group of mice treated with CH_5-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 CH_5-PAA. This strongly support that CH_5-PAA-BNIPDaoct formulation is at least as efficient as gemcitabine. Indeed there is no statistical difference between the CH_5-PAA-BNIPDaoct formulation and gemcitabine.

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 are either ineffective or effective for only 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, 31, 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 apoptosis-inducing effect of Trichostatin (TSA) and suberoylanilide hydroxamid (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, bisnaphthalimidopropyl (BNIPDaoct) could induce apoptosis of various human pancreatic cancer cell lines *in vitro* (37, 38). 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 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 in our previous work (39, 40). 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 CH_5-PAA, an aqueous formulation of CH_5-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 CH_5-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 BNIPDoact molecules (41). The polydispersity index was slightly lower for the CH_5-PAA-BNIPDaoct formulations (0.167) than for the Ch_5-PAA (0.190), this indicated the drug loaded aggregated were of a more uniform size distribution. The size of 178nm is ideal since it has been reported the size of <183nm 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 (BxPC3) cells were exposed to the novel CH_5-PAA-BNIPDaoct formulation *in vitro*, they possessed a notably lower IC_{50} (1.06 μM) than that of commercially available gemcitabine (8.50 μM) demonstrating that BxPC3 cells are more sensitive to the cytotoxic effect of the polymer-drug formulation (Fig. 3). The study was also carried out with the free drug dissolved in DMSO. The difference between the IC_{50} of the free drug and the CH_5-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 CH_5-PAA at the concentrations tested (Fig 3). 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 CH_5-PAA-BNIPDaoct formulation and BNIPDaoct alone confirm that both caused cell death by apoptosis. This is demonstrated by substantial phosptatidylserine exposure on the outer cell membrane, high mitochondrial depolarisation and large increase in DNA fragmentations (Fig 4-6). Unlike polymer, drug formulation and BNIPDaoct alone, gemcitabine at 10μM did not significantly increase the apoptosis and mitochondrial membrane depolarisation in cells compared to the positive controls (Fig 4-6). However, gemcitabine showed substantial DNA fragmentations (Fig 6) indicating the cytotoxic effect of gemcitabine is different from BNIPDaoct and the formulations. CH_5-PAA alone did not cause any substantial increase in apoptosis and mitrochondrial membrane depolarisation in cells (<20%) and negligible DNA fragmentation was observed (<5%), corresponding with the non-cytotoxic effect observed in Fig 3.

The CH_5-BNIPDaoct formulation (1 $mgmL^{-1}$, 0.3 $mgmL^{-1}$) was administered *in vivo* to nude tumour bearing mice over a 4 week period. The study found that the Ch_5, BNIPDaoct formulation was capable of reducing tumour growth rate when compared to CH_5 alone. Although the BNIPDaoct dose used in the formulation is 8-fold less than gemcitabine, the reduction in tumour growth was comparable to the current commercially available drug gemcitabine for anticancer treatment. The reduction in tumour size was significant using the CH_5-PAA-BNIPDaoct formulation ($p< 0.05$) compared to the polymer alone CH_5-PAA. It has been reported that glycol chitosan modified with sterol structure, cholanic acid was able to entrapped docetaxel with the particle size of 320nm and exhibited higher antitumour efficacy in lung cancer cells bearing mice than free docetaxel. The authors showed reduced *in vivo* anticancer drug toxicity compared to free docetaxel and attributed this effect due to the ability of nano-size particles to preferentially localised 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-4 T cell lymphoma compared to other alkyl chains substituents such as dodecyl and oleic acid (41). 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 (CH_5-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 178nm 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, BxPC3 while the polymer itself was non-cytotoxic. Despite the BNIPDaoct dose was 8-fold less than gemcitabine, the formulation was able to reduced tumour growth in xenograft mice with results comparable to gemcitabine. The results of this study indicate that CH_5-PAA-BNIPDaoct has great potential as a suitable therapeutic alternative to gemcitabine for treatment of pancreatic cancer.

Conflict of interest statement

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

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