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A nano-enabled cancer-specific ITCH RNAi chemotherapy booster for pancreatic cancer

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Abstract

Gemcitabine is currently the standard therapy for pancreatic cancer. However, growing concerns over gemcitabine resistance mean that new combinatory therapies are required to prevent loss of efficacy with prolonged treatment. Here, we suggest that this could be achieved through co-administration of RNA interference agents targeting the ubiquitin ligase ITCH. Stable anti-ITCH siRNA and shRNA dendriplexes with a desirable safety profile were prepared using generation 3 poly(propylenimine) dendrimers (DAB-Am16). The complexes were efficiently taken up by human pancreatic cancer cells and produced a 40-60% decrease in ITCH RNA and protein expression in vitro (si/shRNA) and in a xenograft model of pancreatic cancer (shRNA). When co-administered with gemcitabine (100 mg/kg/week) at a subtherapeutic dose, treatment with ITCH-shRNA (3x 50 mg/week) was able to fully suppress tumour growth for 17 days, suggesting that downregulation of ITCH mediated by DAB-Am16/shRNA sensitizes pancreatic cancer to gemcitabine in an efficient and specific manner.

From the Clinical Editor: Gemcitabine delivery to pancreatic cancer often results in the common problem of drug resistance. This team overcame the problem through co-administration of siRNA and shRNA dendriplexes targeting the ubiquitin ligase ITCH.

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Cancer causes over 8.2 million deaths world-wide, a figure set to rise to 12 million by 2030 [1]. For cancers such as pancreatic cancer, 5-year survival rates are as low as 5% because of diagnosis at an advanced stage [2]. Currently, treatment relies mostly on systemic therapy using chemotherapeutic agents with limited specificity. In advanced pancreatic cancer, the cytidine analogue gemcitabine (2′2′-Difluoro-2′-deoxycytidine, GEM) remains the standard course of treatment either in combination with a number of other cytotoxic agents or as monotherapy for patients with poor performance status [3,4].

Recently, molecular-targeted agents have shown considerable potential. These type of agents can be highly specific which potentially reduces side effects, but also limits their applicability to patient populations pre-selected based on the molecular make-up of the individual cancer [5]. As cancer agents are typically used in combination to minimise the risk of drug resistance and dose limiting toxicity, it would be desirable for any newly developed therapy to work well in conjunction with other currently used drugs. Novel forms of therapy that selectively sensitise cancer cells

to the currently used cytotoxic agents would potentially fulfil these requirements and combine improved efficacy, specificity and reduced risk of resistance.

The exploitation of cancer specific molecular targets such as HSP90 or Bcl-2 potentially offers an attractive strategy to improve sensitivity and selectivity of conventional chemotherapeutic agents [6,7]. Similarly, up-regulation of p73, a member of the p53 family of transcription factors, sensitises cancer cells to a variety of chemotherapeutic agents [8,9]. Both p73 and p53 are thought to be involved in promoting apoptosis in response to DNA damage [10]. Consequently, increased levels of these molecules should sensitise cancer cells to cytotoxic drugs that also trigger apoptosis. Although details of p73 regulation in different cancers are still emerging, it is a promising target as, in contrast to p53 ($> 50\%$ mutation) it is rarely mutated [11].

Our group has previously shown that gene therapy using expression of a minimal p53-derived apoptotic peptide can de-repress p73, causing p73-mediated gene activation and tumour regression [12]. RNAi has the ability to selectively switch off certain genes at post-transcriptional level [13]. Here, we evaluate interfering RNA (RNAi) targeting the p73 pathway as a chemo-sensitisation strategy. This is achieved by reducing expression of the ubiquitin ligase ITCH which controls p73 levels by ubiquitination and subsequent targeting to the proteasome for degradation. It is hypothesised that down-regulation of this suppressor would sensitise cells to chemotherapeutic agents and that RNAi based therapy would act as a booster for conventional chemotherapeutic agents [8,9].

In the current study we evaluate small-interfering (siRNA) or short-hairpin RNA (shRNA) as mechanisms for down-regulation and deliver these using a synthetic vector based on generation 3 polypropylenimine dendrimer ('DAB-Am16'). We address the question whether the DAB-Am16 vector system could deliver RNAi based therapies to solid tumours at levels sufficient to allow exploitation of the exquisite specificity of these agents in combination with current anti-cancer agents for improved therapy against challenging cancers such as pancreatic cancer.

Methods

siRNA, shRNA and dendriplexes

siRNAs (*i.e.* anti-ITCH1 5'-3': AAGTGCTTCTCAGAAT GATGA; anti-ITCH2 5'-3: AACCACAACACACGAATTACA at a ratio of 1:1; scrambled control sequence 5'-3': GCAAACCAC CAAUCUAACA; FITC-siRNA 5'-3': AGGUAGUGUAAUCGC CUUG-FITC, all Eurofins MWG Operon, Germany), shRNA (pSUPER1198: CTGCCGCCGACAAATACA Oligoengine, USA, or pSUPER.Mamm-X or a scrambled pSUPER1198 sequence non-specific control) were complexed with DAB-Am16 (Sigma-Aldrich, Dorset, UK) in 96-well plates by mixing equal volumes (100 μL) of solutions of nucleic acid (shRNA or siRNA; 0.5 mg/mL in water) and DAB-Am16 (2.5 mg/mL, dextrose 5% (w/v), pH adjusted to 9 for a degree of protonation (α) = 0.4. Particle size and zeta potential of the resulting dendriplexes were determined on a Nano-ZS Zetasizer (Malvern Instruments, Malvern, UK) at room temperature. Transmission electron microscope (TEM) imaging of particles occurred on a formvar resin-coated copper

grid counterstained with 1% uracil acid (FEI CM120, Biotwin, Eindhoven, Netherlands). Stability of the dendriplexes was evaluated both at 4 °C, under storage conditions and in simulated biological medium at 37 °C.

Cell culture

Pancreatic cells MIA PaCa-2 and PANC-1 (DMEM, 10%FBS, 1% glutamine), HPAC (F12 Ham, 10% FBS, 1% L-glutamine, 1% non-essential amino acids, 2 μg/ml of insulin, 5 μg/ml transferrin 10 ng/ml epidermal growth factor (EGF) and 40 ng/ml of hydrocortisone), and BxPC3 (RPMI-1640, 10% FBS, 1% L-glutamine and 1 mM sodium pyruvate) were obtained from ATCC or ECACC and grown at 37 °C under 5% CO_2, 95% humidity. Cytotoxicity was measured as the IC50 using a MTT based assay 24 hours after the end of a 4 h incubation with free and shRNA complexed DAB-Am16 [14].

Haemolysis assay

Fresh mouse/rat erythrocytes were centrifuged and washed with PBS (1000 g, 10 min at 4 °C for 3 cycles) to produce a 3% suspension which was incubated 1:1 with 5% dextrose containing dendrimer or dendriplex concentrations at 37 °C for 4 hours. Haemoglobin release in the supernatant was determined spectro-photometrically (λ = 570 nm) and compared to negative (dextrose 5%) and positive controls (2% Triton X-100).

Cell uptake in pancreatic cell lines

The interaction of dendriplexes with cells seeded on coverslips in 24-well plates was imaged using scanning electron microscopy (SEM, FEI Quanta 200 F, Eindhoven, NL) after treatment with DAB-Am16/shSCR (1 μg shRNA/ well) and fixation in paraformaldehyde (4% in PBS). Fluorescent dendriplexes (siRNA-FITC or shRNA-Cy3, Mirus Bio LLC, USA) were observed by confocal laser scanning microscopy (CLSM, Leica TCS SP2, Leica Microsystems, UK) after 4 h of incubation and fixation as above. Uptake of dendriplexes with siRNA-FITC was quantified on a LSRII flow cytometer (BD Biosciences, Oxford, UK) and FlowJo software (BD Biosciences) in unfixed cells after 4 h incubation.

ITCH downregulation

Cells were transfected in 24-well plates with DAB-Am16/ shRNA (5 μg shRNA/well in 200 μL of FBS supplemented cell culture medium) or Lipofectamine®2000 as per manufacturer's protocol (Life Technologies, Pasely, UK). After 48 h RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesised using the QuantiTect Reverse Transcription kit. QPCR reactions (10 μl of sam-ple + 15-μL of a PCR-master-mix (QuantiTect SYBR Green PCR kit, Qiagen) used the primer pairs (ITCH QuantiTect Primer Assay) or GAPDH-forward (GGCAGTGATGGCTG-GACTG) and reverse (CGGGAAGCTTGTGATCA ATGG) primers. mRNA levels were evaluated for the target gene (ITCH) and normalised against a reference gene (GAPDH). The cycling conditions were 95 °C for 15 minutes DNA polymerase activation followed by 40 cycles (15" at 94 °C, 30" at 55 °C and 30" at 72 °C) followed by 10 mins at 72 °C. Melting curves

were performed in a range between 65 and 90 °C (data not shown). For mRNA quantification, the amplification efficiency (E) of the target and control genes for each of the transfected conditions was calculated from the slope of a calibration curve established at cDNA concentrations ranging from 10 pg to 1000 ng ($E = 10^{-1/(slope)}$). The ratio (R) of the number of cDNA target molecules over the number of cDNA reference molecules (GAPDH) was calculated as

$$R = \frac{E^{-\text{Ct,target}}}{E^{-\text{Ct,reference}}}.$$

ITCH protein in vitro

Harvested cells (trypsin-EDTA) were repeatedly washed (Dulbecco's PBS) and then lysed (4 °C, 1 h) in RIPA buffer (Sigma-Aldrich) supplemented with phosphatase and protease inhibitor cocktails (Pierce, Thermo Fisher Scientific). Supernatant lysate (15,000 g for 15 min) protein content was assayed using the BCA assay (Pierce). Typically, samples of 30 μg protein were separated by SDS-PAGE (NuPage, Invitrogen; 4-12% poly (acrylamide); 200 V for 50 min) then transferred onto a nitrocellulose membrane (Invitrogen). Blocked membranes (5% non-fat dry milk dispersion prepared in PBS containing 0.1% Tween 20) were incubated (2 h at room temperature or overnight at 4 °C) with primary anti-ITCH antibody (611199, BD Biosciences) or Actin antibody (ACTN05 C4; Abcam, Cambridge, UK), and after five wash steps, for 1 h with the secondary antibody conjugated with horseradish peroxidase (Invitrogen). After 5 washes, blots were developed with SuperSignal west pico chemiluminescent detection kit (Pierce).

In vivo studies

Experimental work was carried out in accordance with local ethics board and UK Home Office regulations and recommendations of UKCCCR where applicable [15]. Dose ranging pilot studies in mice female (CD-1, Harlan Laboratories, Derby, UK or Balb-c Charles River Laboratories Intl, Margate UK) and Sprague-Dawley rats (Harlan Laboratories). Animals were dosed with DAB-Am16 equivalent (mg/kg) in dextrose 5% (w/v) (pH 9 (α = 0.4) or 11 (α =0.1). Body weight and behaviour were recorded [16]. In the absence of symptoms the dose was increased step-wise. The effects of repeat administration and combination regimes were then evaluated at the highest safe single dose level. For tumour studies pancreatic tumour cells (5x10^6) dispersed in 50% growth media/50% Matrigel® (BD Biosciences) were subcutaneously injected in the left flanks of each mouse (100 μL/flank, Swiss nude mice, Charles River). Tumour growth was quantified by serial calliper measurements, body weights were recorded and tumour volumes were calculated (V = πd^3/6).

ITCH tumour down-regulation

Excised MIA PaCa-2 xenograft tumours were stored in RNA*later*® at -80 °C. Tumours were homogenised on ice in 1 mL of lysis buffer containing β-mercaptoethanol using a Polytron® homogeniser (PT MR2100, Kinematica AG, Switzerland) and RNA extracted (Absolutely RNA Miniprep Kit, Agilent, Workingham, UK). Statistical analysis was performed using Origin 6.0. using a two-sample t-test; $p < 0.1$ was considered statistically significant.

Antitumour activity of a combined therapy based on shRNA dendriplexes and GEM in mice bearing MIA PaCa-2 xenografts

Established MIA PaCa-2 tumours ($\simeq 75$ mm^3) were treated with cycles consisting of one IV injection of shRNA dendriplexes (2 mg/kg shRNA; 10 mg/kg DAB-Am16) every other day (days 1, 3 and 5 of a treatment cycle), and GEM (100 mg/kg, i.p.) on day 7 followed by a break of seven days. After statistical analysis of the logarithmic transformed data (two-way analysis of variance, followed by Bonferroni ad-hoc test) values of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered statistically significant (Prism 5.0, Graph Pad Software Inc., La Jolla, CA, USA).

Results

DAB-Am16 dendrimer forms colloidally stable complexes with siRNA and shRNA which can be stabilised further after protein binding.

Creation of stable dendriplexes with a small hydrodynamic diameter requires careful control of the protonation stage by keeping the pH of the dendrimer solution between 6 and 9 (α between 0.8 and 0.4) [17]. We hypothesised that a pH of 9 would strike an adequate balance between the strength of the electrostatic interaction and the potential toxicity of the cationic groups. At this pH, the ionised primary amino groups (pKa 10) at the periphery will be exclusively responsible for complexation with the anionic agents [18]. Under these conditions small positively charged complexes (Figure 1, A) with low polydispersity could be formed with shRNA (133 ± 3 nm, PDI 0.2, ζ 24 ± 5 mV) or siRNA (111 ± 4 nm, PDI 0.2, ζ 27 ± 4 mV). At 4 °C, after an initial size increase (20 nm over 48 hours), the shRNA complexes remain stable with low PDI for at least 14 days while the siRNA particles continue to grow to reach an average diameter of 370 nm. This reduced stability was mirrored by the increased polydispersity observed for the siRNA complexes (Figure 1, A).

While the morphology of the complexes appears similar, these results suggest RNAi molecular weight (shRNA 3176 bp; siRNA 21 bp) to be a key factor in determining stability. The rigidity or length of the nucleic acid may affect complex compactness or change the ability for nucleic acids to bridge particles as shown for inter-polyelectrolyte complexes [19,20].

To evaluate colloidal stability under conditions more representative of physiological environments, the more stable shRNA complexes were further exposed to media containing salt and proteins to simulate in vitro (plain and FBS-supplemented MEM) and in vivo (plain and FBS-supplemented PBS) conditions (Figure 1, B). Exposure to high ionic strength isotonic solutions, in the absence of proteins, resulted in a 4 to 5-fold increase in size after only 5 minutes. This effect could be linked to modulation of the dendrimer-nucleic acid electrostatic interactions after charge neutralisation [21,22] or loss of screening of repulsive electrostatic interactions between particles [23]. Interestingly, the presence of
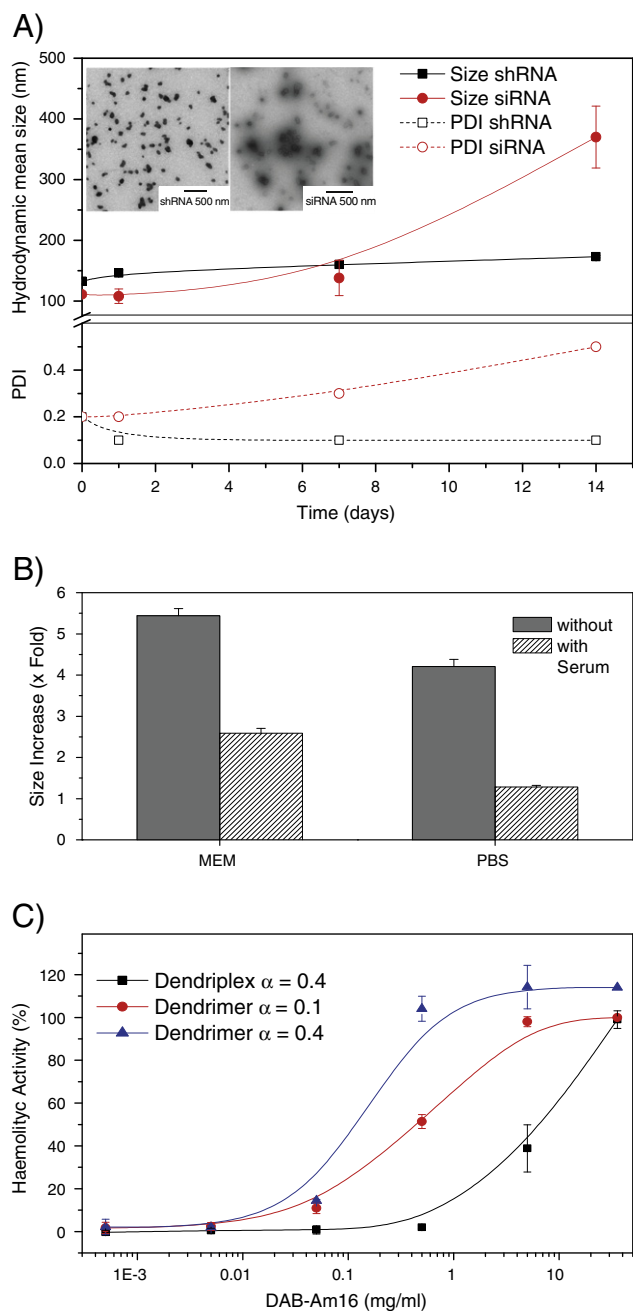
Figure 1. Stability and toxicity of siRNA and shRNA dendriplexes. **(A)** The hydrodynamic mean size and polydispersity of DAB-Am16 complexed with siRNA (21 bp) or shRNA (3176 bp) was evaluated over time at 4 °C. Insert images show TEM of complexes (Left: ▪ shRNA, right: ● siRNA). **(B)** Comparison of the colloidal stability of shRNA dendriplexes in physiological buffers (MEM and PBS) without (grey columns) and with FBS-supplementation (dashed columns). **(C)** Haemolytic activity of DAB-Am16 at pH 11 (α = 0.1; closed circles), pH 9 (α =0.4; closed triangle) and of DAB-Am16/shSCR dendriplexes (α =0.4 used for complexation; closed squares). Hemoglobin leakage was assessed spectrophotometrically after 4 h of incubation at 37 °C. Data represent the average of 3 separate experiments.

FBS under the same conditions dramatically reduces aggregation in both media. This process would most likely occur in the same fashion on injection in vivo with serum proteins rapidly coating

these non-stealth cationic particles after systemic administration [17]. Interactions with serum proteins frequently destroy complexes i.e. when partial coating of the particles accelerates aggregation through a bridging mechanism [24,25]. In contrast, total coating of the particles can actually stabilise particles under physiological conditions [26]. Our data suggest the interaction of proteins with DAB-Am16 dendriplexes leads to a rapid and complete stabilising coating which may play a role in the in vivo efficacy of these dendriplexes after intravenous administration and may play a role in their highly specific distribution [27].

DAB-Am16 complexes efficiently transfect human pancreatic cancer cells

The internalisation of RNAi-based dendriplexes was visualised by SEM (Figure 2, *A*) showing localised membrane pit formation suggestive of uptake along an endocytotic pathway. Consistent with this are the observations following the internalisation using CSLM (Figure 2, *B*). The fluorescent siRNA can be observed in the cytoplasm and perinuclear areas. By contrast shRNA, can be detected in the cytoplasm and nucleus. These studies thus confirm qualitatively the delivery of the nucleic acid cargo to the relevant intracellular compartment. Current evidence suggests the specific pathway (*e.g.* pinocytosis, clathrin-dependent or clathrin-independent/caveolae-mediated) may depend on the transfection agent and cell lines and may affect transfection efficiency [28]. Caveolae and clathrin mediated endocytosis have been shown for polyamidoamine (PAMAM) dendriplexes [29] the latter correlated with higher silencing activity [30]. Dendriplex internalisation was further quantified by flow cytometry, confirming that DAB-Am16 dendrimers were able to deliver the associated siRNA efficiently to a high proportion of cells in all pancreatic cell lines (Figure 2, *C*). In fact, the dendrimer system was significantly more effective at delivering siRNA (80%) than the commercial Lipofectamine® 2000 (20-25%).

Efficient anti-ITCH RNAi down-regulation of target gene expression in pancreatic cancer cells

The previous results demonstrate effective delivery of plasmid- and siRNA-based nucleic acids into cells. In order to established whether these systems were equally suited to achieve downregulation of the target genes their down-regulation of the target gene ITCH was quantified in pancreatic cancer cells (MIA PaCa-2) at the protein and gene expression level (Figure 3, *A* and *B*) demonstrating an ITCH knock-down of 40-60%. Taken as a whole, these results confirmed DAB-Am16 dendrimers to be successful transfecting agents in pancreatic cells for both types of RNAi agents. Based on the fact that better long-term colloidal stability and the evidence for the prolonged duration of silencing effects reported for shRNA [31] we chose to utilise shRNA as the basis for our RNAi ITCH downregulation approach.

The safety of DAB-Am16 depends on protonation state, complexation and dose

Earlier studies have demonstrated that biocompatibility could be a cause for concern for transfection agents such as the DAB-Am16 dendrimer [32]. To test the hypothesis that free dendrimer rather
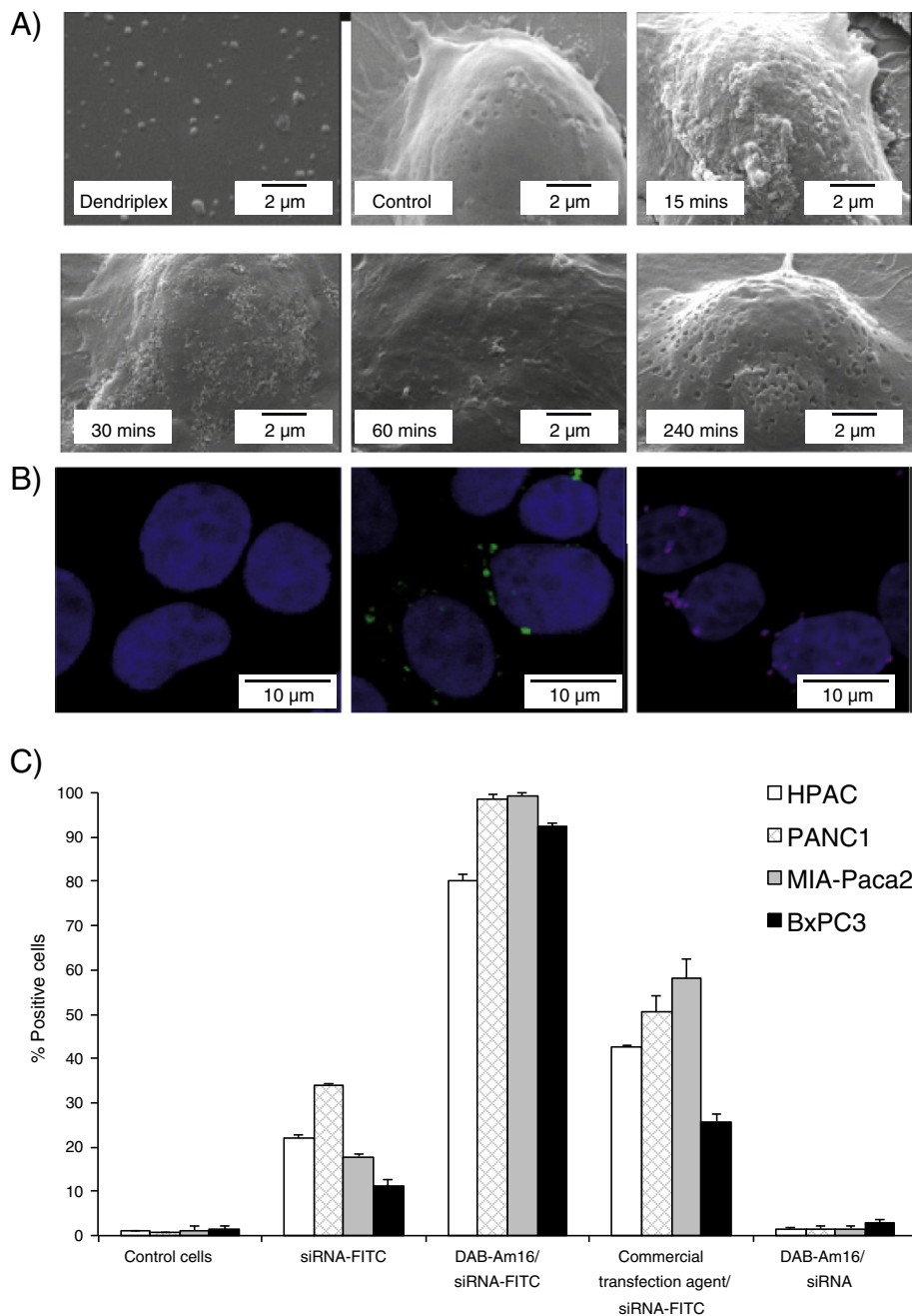
Figure 2. Cell uptake and intracellular trafficking of DAB-Am16/RNAi dendriplexes. **(A)** SEM imaging of the interaction between DAB-Am16/shRNA dendriplexes and MIA PaCa2 cells. Dendriplexes were incubated with the cells for 15 min then washed out. Images were taken prior to (control) and 15, 30, 60 and 240 min after incubation. **(B)** Intracellular localization (at 240 min) of DAB-Am16/siRNA-FITC (green channel) and DAB-Am16/shRNA-EMA (red channel) dendriplexes as probed by CLSM. Cell nuclei are counterstained with DAPI (blue channel) **(C)** Flow cytometric quantification of (at 240 min) cellular internalization of dendriplexes prepared with fluorescent siRNA (DAB-Am16/siRNA-FITC) in various pancreatic cancer cell lines (1) HPAC, (2) PANC1, (3) MIAPaCa2, (4) BxPC3. To disregard unspecific signal, dendriplexes prepared with non-fluorescent RNAi were used as control (DAB-Am16/siRNA).

than complexed material is the source of this toxicity the effect of free DAB-Am16 (solution) was compared to that of DAB-Am16 complexes with scrambled shRNA. The IC50 of DAB-Am16 is cell-type dependent, ranging from the least sensitive BxPC-3 cells (0.14 mg mL^{-1}) to the most sensitive MIA PaCa-2 cells (2 μg/mL) (Table S1). Importantly, equivalent concentrations of dendrimer were less toxic by two to three orders of magnitude, when given in the form of nucleic acid complexes. In order to test the link between

dendrimer toxicity and the presence of cationic groups interaction with murine erythrocytes was compared at a protonation states of $\alpha = 0.1$ and $\alpha = 0.4$ (Figure 1, *C*). At pH 11 the dendrimer has little protonation ($\alpha = 0.1$) making it 5x less haemolytic than at pH 9 ($\alpha = 0.4$). These results are in line with the IC$_{50}$ values obtained; the reduction in toxicity can be attributed to the reduction in the number of free cationic groups available to interact with the anionic erythrocytes membrane after
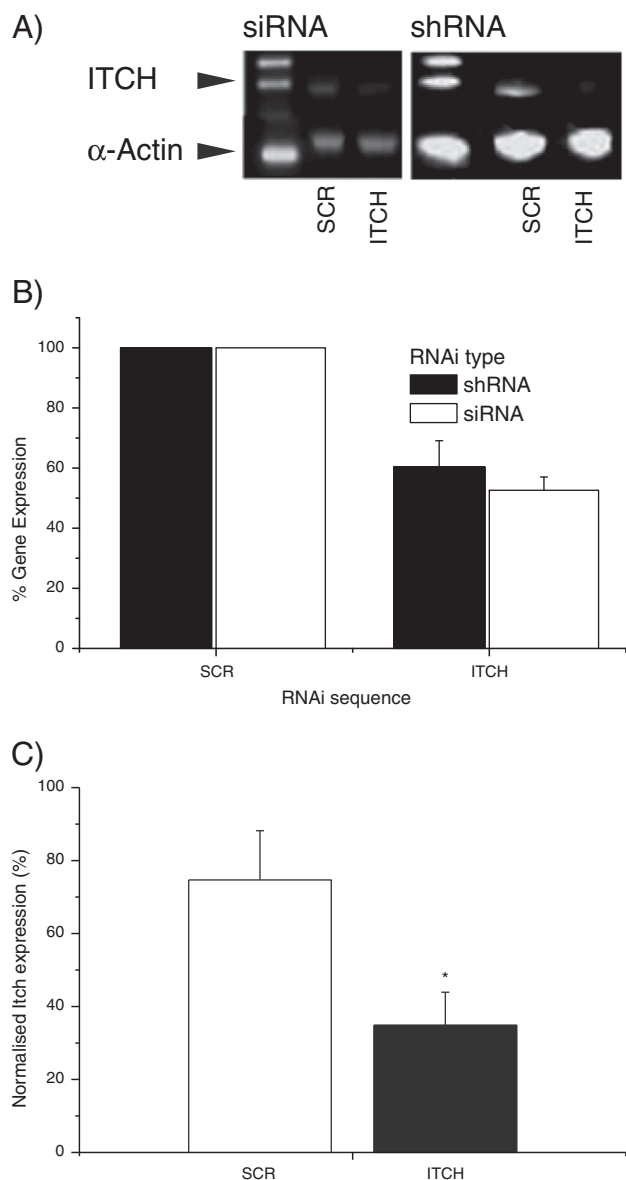
A)

B)

C)

Figure 3. Downregulation of ITCH by DAB-Am16/RNAi. **A)** ITCH protein downregulation in MIA PaCa-2 cells. Western blot showing ITCH protein levels 48 h after transfection with DAB-Am16 dendriplexes (80 μg siRNA or shRNA) carrying scrambled (SCR) or ITCH targeted (ITCH) RNAi. **B)** ITCH mRNA levels in MIA PaCa-2 cells 72 h after anti-ITCH RNAi treatment. Cells were transfected with DAB-Am16 complexed with a control (SCR) or ITCH-targeted (ITCH) RNAi and ITCH levels quantified by qPCR. **C)** Downregulation of ITCH in MIA PaCa-2 tumour xenografts. Tumour-bearing mice received an IV bolus of control shRNA (white bar) or ITCH-targeted (black bar) DAB-Am16 dendriplexes (10 mg/kg dendrimer eq. dose) on day 1, 3 and 5. Data represent mean ± SEM of three replicates. * p < 0.1.

complexation with the nucleic acid [33]. Similarly, complexation of DAB-Am16 also reduces the number of available cationic charges and reduces haemolytic activity by almost an order of magnitude, again. Erythrocytes could be incubated with dendriplexes at concentrations up to 1.25 mg/mL before any haemolytic activity was observed. Interestingly, up to a concentration ca. 1 mg/mL, dendrimers at pH 11 showed lower

haemolytic activity when incubated with rat, rather than murine, erythrocytes, highlighting interspecies differences in toxicological evaluations (Supplemental Figure 1).

Based on haemocompatibility and a projected 10-fold dilution after IV injection, free DAB-Am16 was administered at a starting dose of 10 mg/kg in a pilot study. In mice, dendrimer toxicity, as a single IV bolus, was found to be dose and pH dependent confirming the predictive value of some of the in vitro studies (data not shown). Free DAB-Am16 was well tolerated at doses up to 30 mg/kg when administered as a 3 mg/mL solution (pH 9). An increase of the pH to 11 allowed the administration of doses up to 30 mg/kg without toxicity. On the other hand, a slight dilution of the dendrimer (from 4 mg/mL to 2.5 mg/mL) and a slow rate of injection without altering the pH, did not improve tolerability, confirming the importance of controlling ionisation. Encouragingly, repeated daily administrations of the free dendrimer at a dose of 10 mg/kg over 5 days (cumulative dose of 50 mg/kg) did not result in significant weight loss and clinical signs of toxicity were not observed. In rats, free DAB-Am16 could be safely administered at doses up to 40 mg/kg. This finding also correlates well with the haemocompatibility results (Supplemental Figure 1) where rat erythrocytes were shown to better resist the action of the dendrimers confirming the importance of species differences. To allow the use of relevant human pancreatic tumour cell lines as the disease model we proceeded to use mice for further studies.

Finally, the safety of combination of shRNA dendriplexes with GEM was evaluated. GEM currently constitutes the first line of treatment for pancreatic cancer [3]. Typical regimes involve GEM therapy in combination with paclitaxel, erlotinib, capecitabine or cisplatin. In patients with poor performance status, monotherapy is typically given to minimize the potentially detrimental side effects associated with more aggressive treatment regimes. GEM (up to 1000 mg/m^2) is given as a cycle consisting of weekly 30-minute infusions spread over three weeks. In our study, GEM was administered weekly as a single IP injection of a 50 mg/kg or 100 mg/kg dose. Based on dose ranging studies, a treatment schedule for the shRNA combination therapy was devised consisting of three IV administrations of dendriplexes on alternate days (days 1, 3 and 5) with GEM following on day 7. Co-administration of GEM with the dendriplexes (day 1) or between DAB-Am16/shRNA injections (day 2) proved toxic but interestingly, the combination was well tolerated even over several cycles when the initial dose of GEM was administered on day 7, irrespective of the GEM dose.

DAB-Am16/shRNA reduces expression of ITCH target protein in pancreatic tumour xenografts

The ability of DAB-Am16 dendrimers to deliver an ITCH-targeted shRNA to pancreatic tumours and for the shRNA to affect an effective down-regulation of the ITCH target gene was studied in mice bearing MIA PaCa-2 xenografts (Figure 3). To this end, the dendriplexes were administered on alternate days for three days at a dose equivalent to 10 mg/kg of DAB-Am16. Intratumoural ITCH mRNA levels were determined on day 6, i.e. 24 h after the last dendriplex injection, and revealed 40% decreased mRNA levels compared to tumours treated with the scrambled control shRNA (Figure 3, C). Given the fact that the harvested tumour contains a range of non-cancer cells, e.g. vascular and connective tissue, this value is very consistent with the effects observed in the pancreatic
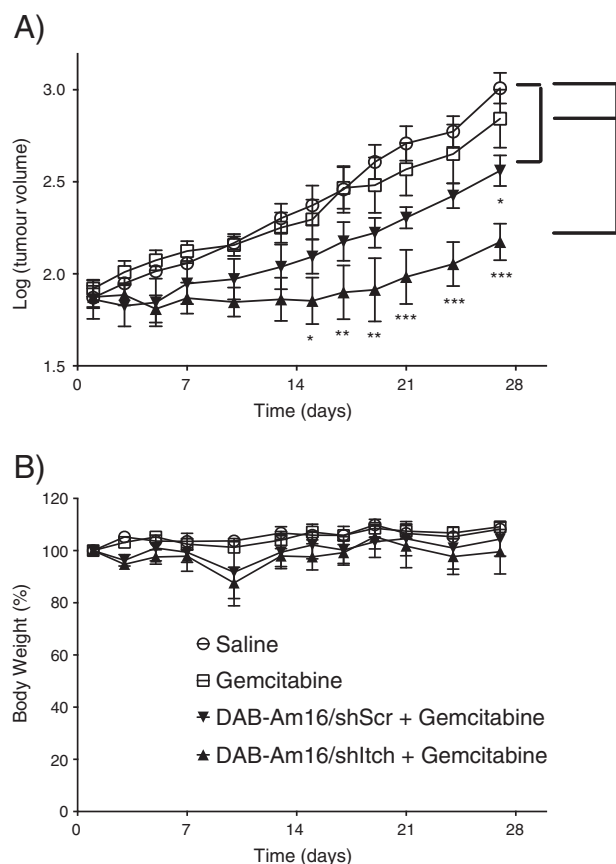
Figure 4. Efficacy of dendriplex/GEM combination therapy. **(A)** Tumour growth of MIA PaCa-2 xenografts control mice (open circle, saline) and mice treated with GEM (open square, 100 mg/kg, days 7 and 21), and GEM in combination with DAB-Am16/shSCR (inverted closed triangle, 10 mg/kg DAB-Am16 and 2 mg/kg shSCR, on days 1, 3, 5, 15, 17, 19), or Gemcintabine in combination with DAB-Am16/shITCH (closed triangle, 10 mg/kg DAB-Am16 and 2 mg/kg shITCH, days 1, 3, 5, 15, 17, 19). **(B)** Body weight of mice served as a surrogate marker of toxicity and was recorded throughout the experiment. Saline treated animals were used as control. Graphic represents mean ± SEM (n = 4). Statistical differences are represented as * ($p < 0.5$), ** ($p < 0.05$), and *** ($p < 0.001$).

cancer cell lines (Figure 3, A-B) and suggests that transfection is a relatively efficient process.

DAB-Am16/shRNA in combination with GEM efficiently slows pancreatic tumour progression

In order to test whether the levels of downregulation observed could potentially improve therapeutic outcome in pancreatic cancer, the RNAi therapy was evaluated in combination with GEM (Figure 4). Mice carrying established MIA PaCa-2 xenograft tumours (>5 mm ø) were treated with two cycles of a combination of anti-ITCH shRNA dendriplexes (10 mg/kg DAB-Am16 and 2 mg/kg shRNA on day 1, 3, 5) and GEM (100 mg/kg, day 7).

GEM, an anti-metabolite drug of the nucleoside-analogue type, is frequently used in advanced pancreatic cancer because it is well tolerated [3]. GEM, when administered alone at a dose of 100 mg/kg, had no significant impact on tumour growth in our model, allowing tumours to reach more than 10-times the starting

volume within 24 days (Figure 4, *A*). Similar results have been reported for GEM in MIA PaCa-2 models at a similar dose, and improved efficacy has been previously reported in combination with other therapeutic agents, including nucleoside analogues [34], flavonoids [35] or co-treatment with monoclonal antibodies and radiation [36].

p53, is a tumour suppressor gene involved with the induction of cancer cell apoptosis in response to chemotherapy. However, more than 50% of cancers have been shown to have disabled this pathway and in pancreatic cancers the rate of p53 mutations could be as high as 70% [37]. In contrast, the pro-apoptotic protein p73 is rarely mutated and potentially offers a functional alternative [38] that we have previously shown can be exploited therapeutically [12]. Here we have chosen to test whether down-regulation of ITCH, an E3 ubiquitin ligase that targets the pro-apoptotic protein p73 for degradation could be used to sensitize pancreatic cancers to conventional chemotherapeutic agents.

While the combination of GEM and scrambled shRNA dendriplexes has some effect, this was significant ($p < 0.5$) only on day 27 (Figure 4, *A*). This observation is not entirely surprising as an inhibitory effect of DAB-Am16 on the growth of some tumours has been previously reported [39].

In contrast, the combination of the ITCH-targeted shRNA dendriplexes and GEM caused a highly significant delay ($p < 0.001$) in tumour growth, apparent from the second treatment cycle (Figure 4, *A*). While the tumours of untreated animals and those on GEM monotherapy or scrambled shRNA/GEM combination therapy progressed within a week, the animals treated with the ITCH shRNA/GEM combination showed stable disease for 17 days. The untreated tumours grow 14-fold in volume within four weeks compared to the animals treated with the ITCH shRNA/GEM combination, which only doubled in size. Importantly, the treatment was very well tolerated with no significant differences in body weight observed between treatment groups (Figure 4, *B*). The combination of lack of toxicity and high efficaciousness suggests that the downregulation of ITCH does indeed lead to a tumour-specific sensitisation to GEM, which by itself does not exhibit activity against this pancreatic tumour.

Discussion

The promise of RNAi for cancer treatment has led to the development and subsequent clinical evaluation of a variety of agents providing proof-of-principle for this form of therapy [40]. Still, efficient systemic delivery remains a major obstacle, preventing wider use of RNAi-based therapies, in particular for therapy of advanced cancer [41]. Encapsulation of RNAi agents in nano-sized vectors by complexation of the anionic nucleic acids to an excess of cationic linear, branched or dendritic polymers is a promising strategy for delivery of genetic therapies [42,43]. The generation 3 poly(propylenimine) dendrimers (DAB-Am16) offer a balance between safety and high transfection efficiency in vitro and in vivo [32,39]. While the polymer has previously been used for TNFa based cancer gene therapy we wanted to explore whether these systems could also be effective for RNAi based therapies. We were able to demonstrate that the toxicity of DAB-Am16 is closely linked to its protonation state and that complexation with nucleic

acids reduces cytotoxicity and haemolytic potential by at least two orders of magnitude. These observations also hold true for the in vivo administration of the system. We report that DAB-Am 16 dendrimers form colloidally stable complexes with siRNA and shRNA which are efficiently taken up by endocytotic mechanisms into a range of human pancreas cancer cell lines. The dendriplexes based on siRNA and shRNA achieve effective downregulation of the ITCH target RNA and protein expression in vitro and in a xenograft model of pancreatic cancer (shRNA).

In combination with a therapeutically ineffective dose of GEM (100 mg/kg/week) the ITCH-RNAi treatment (3x 50 mg/week) was able to fully suppress tumour growth for 17 days.

Drug resistance poses a significant clinical challenge, which chemosensitisation strategies aim to address. Cancer resistance mechanisms can be categorized into predominantly pharmacodynamic or pharmacokinetic, i.e. mechanisms that reduce the ability to engage the cancer cell's downstream response (typically apoptosis), or those, which reduce the exposure of the cellular target to the drug (e.g. increased metabolism or efflux pumps etc.) [44]. In pancreatic cancer cells a number of putative pharmacodynamic (e.g. HMAGA1, MUC4, HIF1a, HSP27, c-Scr, c-Met) and pharmacokinetic resistance mechanisms (e.g. hENT1, cDK) have been identified, however, their clinical relevance has not been confirmed in all cases [44]. The clinical evidence suggests reduced GEM transport and exposure as important factors in drug resistance in pancreatic cancer. Barriers to drug transport develop due to pathological features of the neo-vasculature, the stroma and cellular transport proteins; for example, cellular expression of the transport related proteins hENT1 and cDK has been shown to lead to increased patient survival [45] while reduced transport measured using biomarkers has been shown to predict resistance [46].

The use of RNAi therapies that target drug resistance related molecular targets potentially allows a highly specific interventions and chemosensitisation. In lung cancer, RNAi strategies targeting c-myc [47], polo-like kinase I (PLK1, [48]), and ribonuclotide reductase subunit 1 (RRM1, [49]) have shown sensitisation of tumours to GEM.

Re-setting of the cellular apoptotic balance, which has been well-established as the mechanism of action of RNAi mediated ITCH downregulation [8,9,50], would lead to a sensitization of the affected pancreatic cancer cells in a wider range of circumstances. Our observations demonstrate that a combination of GEM with anti-ITCH RNAi can lead to good control of tumour growth in conditions of suboptimal drug exposure, irrespective of whether they are caused by pharmacokinetic or pharmacodynamic mechanisms (Figure 4). Our data suggest this approach could provide an elegant and specific way to increase the efficacy of conventional anti-cancer drugs that would fit well with the current therapeutic approaches to treatment of advanced pancreatic cancer, for which GEM, either alone or in combination with other cytotoxic agents/and or radiotherapy, is currently the treatment of choice [3].

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2014.09.010.

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