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Mucin overexpression limits the effectiveness of 5-FU by reducing intracellular drug uptake and antineoplastic drug effects in pancreatic tumours

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ABSTRACT

Current treatments for pancreatic cancer have failed to effectively manage the disease, and hence, more effective treatment approaches are urgently needed. Studies suggest that mucin *O*-glycosylation limits the cytotoxic effect of fluorouracil (5-FU) against the growth of human pancreatic cancer cells *in vitro*. In the present study, we investigated the relationship between the levels of mucin *O*-glycosylation expressed in pancreatic tumours and the antitumour effect of 5-FU. The inhibition of *O*-glycosylation was achieved by intratumoural (IT) injections of benzyl-α-GalNAc. Immunohistochemical staining of human pancreatic tumours revealed relatively high (Capan-1) and moderate (HPAF-II) expression levels of MUC1 mucin compared to MUC1 negative control (U-87 MG human glioblastoma) tumours. The antitumour effects of 5-FU (given systemically) against Capan-1 tumours improved significantly following IT injections of benzyl-α-GalNAc. Histochemical staining of tumour sections revealed a reduced number of neoplastic cells in tumours exposed to benzyl-α-GalNAc prior to 5-FU treatment compared to 5-FU alone. Furthermore, intracellular uptake of 5-FU by Capan-1 cells was significantly greater following injections of benzyl-α-GalNAc; however, no such effect was observed with U-87 MG cells. Mucin overexpression reduces intracellular drug uptake, antineoplastic and antitumour drug effects, which may have important clinical implications in treatment.

1. Introduction

Pancreatic cancer is the fourth most common cause of cancer-related deaths in the United States (US), with a 5-year survival rate of <5%.[1] The use of a single chemotherapeutic agent (such as infusional 5-FU) or in combination (such as with gemcitabine, along with oxaliplatin, cisplatin and irinotecan) has had little positive impact on overall patient survival.[2] Current research has thus focused on identifying and exploiting cell surface receptors, specific ligands and signal transduction pathways involved in pancreatic cancer and treatment. One such cellular target (given its abundant level of expression in pancreatic tumours) belongs to a series of cell surface glycoproteins called mucins.[3]

Mucins are high molecular weight glycoproteins expressed on the surface of epithelial cells in various organs such as lungs, breast, kidneys, pancreas and other organs in the gastrointestinal tract.[4] The dense network of oligosaccharide chains attached to the protein core contributes to approximately 80% of the total mass of these high molecular weight glycoproteins.[4] The expression profile of 19 mucins identified to date varies as a function of the organ environment;

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however, the most abundantly expressed mucin variety in a particular organ will determine the overall extent to which the dense glycosylation mesh will form.[5]

Under normal physiological conditions, mucins are present at the apical surface of epithelial cells, where they protect the epithelial surface from the external environment and alter cell signalling events.[6] However, a dysregulation in mucin expression is observed in pathological diseases such as cystic fibrosis, chronic obstructive pulmonary disorders and ulcers, and in cancer.[7,8] In cancer cells, the apical expression of mucins is lost, and the expression of mucin completely surrounds the cell surface. To date, studies support the importance of mucin in cancer, including its involvement in protecting cancer cells from the adverse external environment, in assisting cancer cells in evading immune responses and in facilitating cellular signalling events that promote an aggressive disease state.[6,9]

Clinical studies suggest a correlation between the overexpression of mucins in pancreatic cancer patients and poor survival.[10,11] Extracellular-bound mucin was previously shown to impede the cytotoxic effect of 5-FU against the growth of pancreatic cancer cells *in vitro*.[12] However, the influence of mucin *O*-glycosylation on the effectiveness of chemotherapeutic agents in preclinical models has not been established. We thus investigated whether the mucin glycation mesh produced during the normal development of human pancreatic tumours limits the overall effectiveness of 5-FU *in vivo*.

The reagent benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (benzyl-α-GalNAc) was used to inhibit the synthesis of mucin *O*-glycosylation in Capan-1 tumours. The antitumour drug effects were determined by an initial evaluation of tumour response to the various treatment approaches *in vivo*, and were then confirmed by histochemical staining and analysis of the tumour specimens immediately following treatment. The following investigation supports a relationship between the intracellular levels of 5-FU in human pancreatic cancer cells (which is limited by the mucin glycation mesh) and the overall susceptibility of pancreatic tumours to the chemotherapeutic drug effects of 5-FU.

2. Material and methods

2.1. Materials

The chemotherapeutic agent 5-FU and benzyl-α-GalNAc were purchased from Sigma–Aldrich (St Louis, MO). Cell culture media Eagle's minimum essential medium (EMEM), and Iscove's modified Dulbecco's medium and trypsin-EDTA (ethylene-diaminetetra acetic acid) were purchased from ATCC (Manassas, VA).

2.2. Cell culture

The human pancreatic cancer cell lines Capan-1 (HTB-79) and HPAF-II (CRL-1997) were maintained in Iscove's modified Dulbecco's medium and Eagle's minimum essential medium (EMEM), respectively. The human brain cancer cell line U-87 MG (glioblastoma, astrocytoma grade III) was maintained in EMEM supplemented with 10% foetal bovine serum. All cell lines were grown in a humidified CO_2 atmosphere at 37 °C.

2.3. Immunohistochemical staining

Expression of MUC1 levels in tumours was detected by immunohistochemical staining by an immunoperoxidase method.[13] Briefly, 4 μm sections of paraffin-embedded tissues were mounted on glass slides and incubated at 60 °C for 1 h. Each slide was deparaffinised with xylene and rehydrated through a graded series of alcohol concentrations. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide/methanol for 10 min and washed under running tap water for 5 min. The slides were incubated for 1 h with primary anti-MUC1 antibody (NCL-MUC1, Novocastra, UK). Following washing, the slides were incubated with biotinylated secondary antibody and then with avidin–biotin complex-Horseradish peroxidase for 45 min. All incubations were carried out in a humidified chamber. The sections were developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as substrate and counter-stained with Mayer's haematoxylin. The slides were then viewed under a brightfield microscope (Olympus BX61WI, Melville, NY) at 20× magnification.

2.4. Tumour cell implantation and response study

All animal work was performed in the animal facility at Northeastern University, Boston, Massachusetts in accordance with the institutional guidelines. Female severe combined immunodeficient (SCID) mice, 6- to 8-weeks-old, purchased from Massachusetts General Hospital, Boston, Massachusetts, were used in the study, since they have been used successfully for developing human xenografts of pancreatic tumours.[14] To establish the tumours, 2.5×10^6 Capan-1 cells in 0.1 ml of cell culture medium were injected into the subcutaneous dorsa of all mice. Mice were weighed daily using a digital weighing balance (accuSeries®, Fisher Scientific, Arvada, CO), and tumour volumes were measured daily using an electronic digital caliper (Control Company, Friendswood, TX). Tumour volumes were calculated using the formula $a^2 \times b \times 0.52$, where '*a*' is the longer diameter and '*b*' is the shorter diameter. Once the tumour volumes were approximately 50–70 mm^3 the experimental groups received intratumoural injections of benzyl-α-GalNAc (0.1 ml injection; 10 mg/ml), whereas the control groups were given intratumoural saline injections (0.1 ml). A total of four intratumoural injections were given at intervals of 48 h. When the tumour volumes were approximately 100 mm^3, 5-FU was administered via the intravenous route. Animals in treatment groups received two injections (0.1 ml) of 5-FU at an interval of 4 days, whereas control mice received saline (0.1 ml). The total dose of 5-FU administered was 125 mg/kg. At the end of the experiment the animals were sacrificed, tumour tissue was surgically removed and fixed in 10% formalin at 4 °C for histochemical staining and analysis.

2.5. Histochemical staining

Paraffin-embedded tissue sections (5 μm) were stained with haematoxylin and eosin (H&E) to evaluate and compare the

extent of tissue viability. The tumour sections were scanned and qualitative images captured using brightfield microscopy (Olympus BX61WI, Melville, NY). The number of tumour cells in each section was quantified using bioquant imaging software (BIOQUANT Image Analysis Corporation, Nashville, TN) and expressed as percent of total tumour area. The outline of the entire tumour section was first traced in each field of view (FOV). The pixels within the traced area were then selected, and the pixel count was determined using the software. A total of five FOV were analysed to determine the total pixel count of the tumour area. Microscope stage encoders were used to ensure that no tumour area was counted more than once. Similarly, the total pixel count of the neoplastic cells featured in blue in each FOV was determined. The total percent of neoplastic cell density was calculated as follows:

$$\text{Percent cell density} = \frac{\text{Total pixel count for cells (blue) staining}}{\text{Total pixel count for entire tumour area}} \times 100.$$

2.6. Immunolabelling of intracellular 5-FU

Sterile coverslips were placed in 24-well plates (Corning, NY). Cells were seeded at 2×10^4/ml in the same 24-well plates. Following a 24 h incubation period at 37 °C, cells were exposed to the maximum non-toxic concentration of benzyl-α-GalNAc solution prepared in cell culture media[12], the control cells were incubated in growth media without the *O*-glycosylation inhibitor. Following an additional 48 h of incubation, each row was washed with 1X PBS and treated with 5-FU prepared in cell growth medium. The intracellular uptake of 5-FU following 1 and 4 h of cell exposure to drug was determined by immunofluorescence labelling. Briefly, the medium containing 5-FU was aspirated, and cells were washed twice with 1X PBS. The cells were then fixed with cold methanol for 10 min at –20 °C followed by rinsing with cold acetone. The cells were next washed twice with 1X PBS followed by 30 min rehydration in 1X PBS. For immunolabelling, cells were exposed to 200 μl (1:1000 dilution; stock concentration: 1 mg/ml) of primary antibody against 5-FU (Lampire Biological Laboratories, Pipersville, PA) for 1 h at room temperature. The unassociated primary antibody was removed by washing three times with 1X PBS, and cells were then incubated at room temperature with 200 μl (1:1000 dilution; stock concentration: 1 mg/ml) of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody (Lampire Biological Laboratories, Pipersville, PA).

Following 1 h incubation with the secondary antibody, cells were washed twice with 1X PBS to remove any unbound secondary antibody. The fluorescence intensity was measured using a fluorescence microplate reader (Bio-Tek® Instruments Inc., VT) at an excitation wavelength of 485 nm and at an emission wavelength of 528 nm. The intracellular uptake of 5-FU was observed under a fluorescence microscope. The coverslip from each well was mounted onto a glass microslide (Corning, NY) with SlowFade® Gold antifade reagent (Invitrogen, Carlsbad, CA). The images were captured using a combination of fluorescence and DIC microscopic applications at 20× magnification (Olympus BX61WI, Melville, NY).

2.7. Statistical analysis

To determine the significant difference between experimental groups, the non-parametric Mann–Whitney *U*-test was used. ANOVA was used to determine differences between more than two experimental groups. Statistical significance was established at $P \leqslant 0.05$. Analysis was performed using the statistical package SPSS 12.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Evaluating the inhibition of mucin O-glycosylation in pancreatic tumours

The relative abundance of different types of mucins overexpressed in tumours is a function of the specific organ and environment. For example, colon carcinomas overexpress MUC5AC, MUC6 and MUC17, whereas lung adenocarcinomas show high levels of MUC1, MUC3 and MUC4.[5] It has been demonstrated that the two human pancreatic cancer cell lines Capan-1 and HPAF-II show relatively high and moderate levels of MUC1 mucin, respectively; whereas the U-87 MG cells (used as negative control cell line) showed no MUC1 expression.[12] In the present study, we determined the levels of MUC1 expression in Capan-1, HPAF-II and U-87 MG tumours both with and without pre-treatment with benzyl-α-GalNAc. We performed immunohistochemical staining of tumour sections with anti-MUC1 antibody targeting the carbohydrate epitopes of MUC1 protein (Fig. 1). Capan-1 tumours (Fig. 1A-a) showed a high MUC1 staining as compared to a moderate staining in HPAF-II tumours (Fig. 1A-b), whereas U-87 MG tumours showed no staining with anti-MUC1 antibody (Fig. 1A-c). These results confirm MUC1 levels in tumours for Capan-1 > HPAF-II > U-87 MG. The use of benzyl-α-GalNAc reagent as an inhibitor of mucin *O*-glycosylation in pancreatic cancer cells had been demonstrated previously.[12] In order to achieve a similar effect *in vivo* it is important that benzyl-α-GalNAc is available in sufficient concentrations to significantly reduce mucin *O*-glycosylation levels in the pancreatic tumours. Several studies have shown that intratumoural administration of therapeutic agents such as antibodies or cytokines have been successful in maintaining high concentrations of these agents at the local site of injection.[15,16] We thus administered benzyl-α-GalNAc locally into the tumour mass via intratumoural injections. A total of four intratumoural injections were given at an interval of 48 h. Following intratumoural injections, Capan-1 and HPAF-II tumours were analysed for MUC1 carbohydrate staining (Fig. 1B). We observed lower staining for MUC1 *O*-glycosylation in tumours exposed to benzyl-α-GalNAc for both HPAF-II (Fig. 1B-b) and Capan-1 (Fig. 1B-d), when compared to tumours not exposed to the inhibitor (Fig. 1B-a and B-c). Therefore, the intratumoural injections of benzyl-α-GalNAc adequately inhibited mucin *O*-glycosylation.

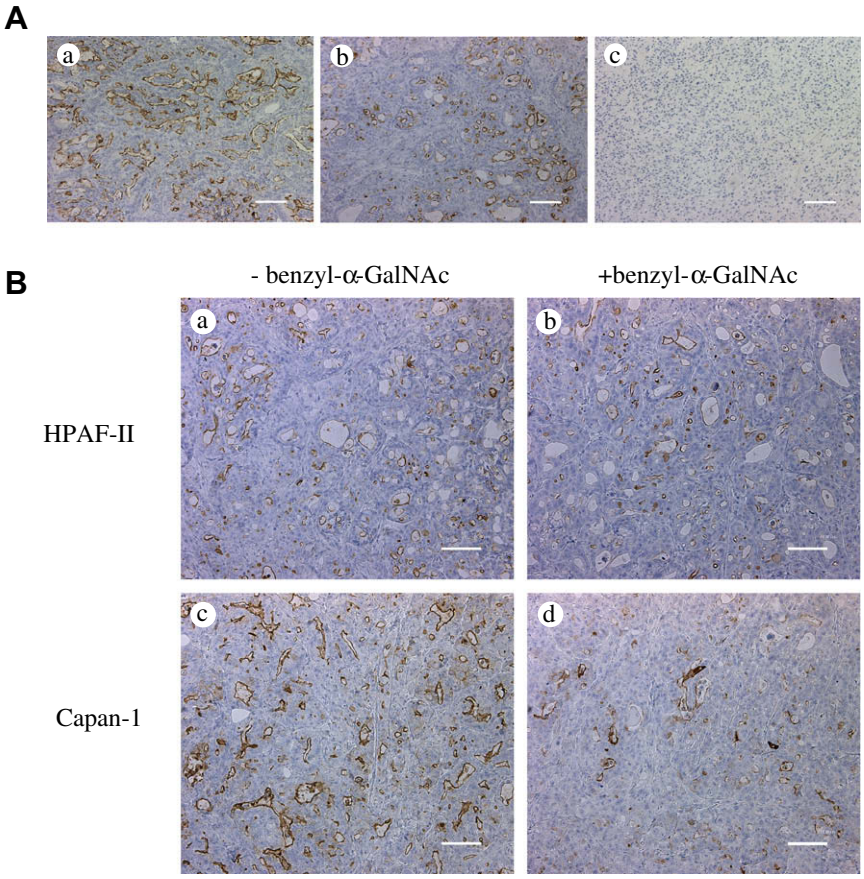


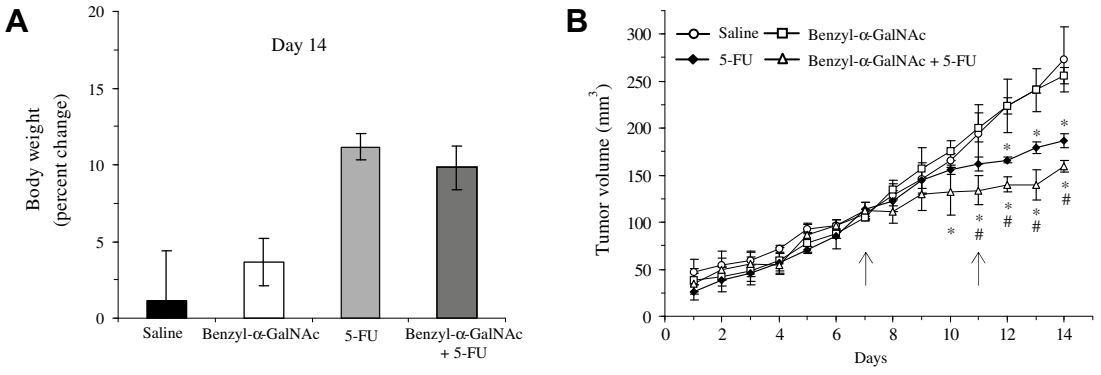
Fig. 1 – Immunohistochemical staining of tumour specimens using anti-MUC1 antibody (NCL-MUC1). A: Relative extent of MUC1 levels (brown staining) in (a) Capan-1, (b) HPAF-II and (c) U-87 MG tumours. B: Effects of benzyl-α-GalNAc on MUC1 O-glycosylation levels in pancreatic tumours. Tumours in treatment group (b, d) received four intratumoural injections of benzyl-α-GalNAc (10 mg/ml, 0.1 cc) at an interval of 48 h, whereas control tumours (a, c) received saline injections (20× magnification; bar: 50 μm).

3.2. *Effects of inhibiting mucin O-glycosylation on antitumour activity of 5-FU*

The ability of pancreatic cancer cells (i.e. Capan-1 and HPAF-II) to respond to 5-FU treatment *in vitro* can be enhanced following the inhibition of mucin *O*-glycosylation.[12] We performed a tumour response study to determine whether reducing the mucin glycation mesh in pancreatic tumours in mice would improve the antitumor activity of 5-FU. For these studies, the pancreatic cancer cell line Capan-1 was used to establish proof-of-concept.

An evaluation of changes in animal body weight in response to treatment was used to assess toxicity. There were no significant changes observed in the weight of the animals in all experimental groups when compared to controls (data not shown), thus supporting a treatment regime that was well tolerated. The experiment continued until day 14 when percent change in the body weights of mice receiving 5-FU treatment was approximately 10% (Fig. 2A). By terminating the study on day 14, we were able to resect tumour masses from all tumour-bearing mice in the study. This was an important goal, since the premature removal of any mice from the study owing to severe toxicity would prevent the direct correlation between the cumulative daily effects of treatment on pancreatic tumour growth (Fig. 2B) and the density of neoplastic cells in the same tumours (Fig. 2D).

The tumour volume was monitored as an indicator of tumour response to 5-FU treatment (Fig. 2B). We observed no difference between the tumour volumes in control animals receiving intratumoural injections of saline when compared to injections of benzyl-α-GalNAc. Therefore, the intratumoural injections of the mucin inhibitor did not alter the rate of tumour growth. The groups that received 5-FU treatment (either 5-FU alone or benzyl-α-GalNAc + 5-FU) had significantly lower ($^{*}P \leqslant 0.05$) tumour volumes as compared to control tumours (saline group), suggesting that the dose of 5-FU was able to suppress the tumour growth. The tumours pre-treated with mucin *O*-glycosylation inhibitor followed by 5-FU treatment (benzyl-α-GalNAc + 5-FU group) showed significantly lower tumour volumes ($^{\#}P \leqslant 0.05$) as compared to 5-FU treatment alone (intratumoural saline + 5-FU). The histochemical staining of tumour sections showed a dense packing of neoplastic cells in both the control groups, receiving either intratumoural injections of saline (Fig. 2C-a) or benzyl-α-GalNAc (Fig. 2C-b). These images suggest that the intratumoural injections of inhibitor were not toxic to the pancreatic tumour at the



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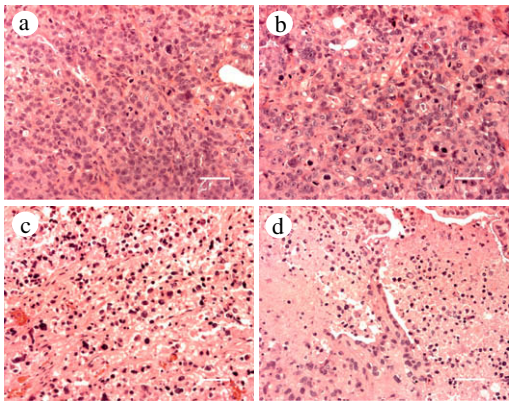

D

Group #	Treatment	Percent tumor area
1	Saline	27.9 ± 9.4
2	Benzyl-α-GalNAc	28.3 ± 1.8
3	5-FU	21.0 ± 1.4 *
4	Benzyl-α-GalNAc + 5-FU	11.4 ± 5.6 * #

Fig. 2 – Effects of inhibiting mucin on antitumour activity to 5-fluorouracil. Capan-1 tumours were established in s.c. dorsa of female SCID mice. When tumour size reached ~50–70 mm^3, intratumoural injections of benzyl-α-GalNAc (0.1 ml, 10 mg/ml) were administered on days 4, 6, 8 and 10; the control groups received comparable injections of saline. 5-FU therapy (↑) began when tumour size was ~100 mm^3. (A) Percent change in body weight on day 14. (B) Tumour growth in response to 5-FU treatment was significantly lower ($^*P \leqslant 0.05$) compared to control groups (saline and benzyl-α-GalNAc). Tumour volumes of group exposed to benzyl-α-GalNAc followed by 5-FU treatment were significantly lower ($^\#P \leqslant 0.05$) compared to 5-FU treatment alone. (C) Histochemical staining of tumour sections showing the arrangement of neoplastic cells within (a) saline, (b) benzyl-α-GalNAc, (c) 5-FU and (d) benzyl-α-GalNAc + 5-FU treated tumours (20× magnification; bar: 50 μm). (D) Quantification of neoplatic cell density using bioquant imaging software. Neoplastic cell density was significantly lower ($^*P \leqslant 0.05$) for 5-FU treated groups as compared to controls. The group exposed to benzyl-α-GalNAc followed by 5-FU treatment had significantly lower ($^\#P \leqslant 0.05$) neoplastic cells compared to 5-FU treatment alone. The values represent mean ± s.d. of four animals.

concentrations employed. The tumours that received 5-FU treatment [5-FU alone (Fig. 2C-c) or benzyl-α-GalNAc + 5-FU (Fig. 2C-d)] showed a fewer number of tumour cells compared to the control tumours. The tumour cells within these sections were also more dispersed throughout the interstitial tumour matrix. Qualitative analysis of histological tumour sections previously treated with benzyl-α-GalNAc + 5-FU revealed a reduced number of neoplastic cells in the tumour sections when compared to the 5-FU control group (Fig. 2C-c and C-d).

We next quantified the neoplastic cell densities (as a percent of total tumour area) within these tumour sections using bioquant imaging software (Fig. 2D). The control groups receiving either intratumoural injections of saline or benzyl-α-GalNAc showed no difference in total cell densities. The tumours treated with 5-FU showed a significantly lower number of tumour cells when compared to control groups ($^*P \leqslant 0.05$). Finally, the neoplastic cell density within the tumours pretreated with benzyl-α-GalNAc followed by 5-FU therapy was significantly lower ($^\#P \leqslant 0.05$) when compared to 5-FU treatment alone. These results support our tumour volume data, which show that the inhibition of mucin *O*-glycosylation in pancreatic tumours enhanced the antitumour effect of 5-FU.

3.3. *Intracellular uptake of 5-FU is enhanced in a reduced extracellular mucin environment*

The effectiveness of 5-FU treatment is a direct result of the successful conversion of 5-FU prodrug to its active metabolites (such as FUrd, FdUrd, FUTP and FdUMP) formed within the intracellular compartments of the cells.[17] The total levels of metabolites formed within target cells will thus depend on the intracellular levels of 5-FU and the various enzymes participating in the conversion of prodrug to active metabolites.[18] We next determined whether the enhanced therapeutic activity of the drug observed *in vivo* (following the inhibition of mucin *O*-glycosylation) was owing to a significant increase in the intracellular levels of 5-FU.

For these studies, we exposed the cells to the maximum non-toxic concentration of benzyl-α-GalNAc for 48 h followed by 1 and 4 h exposure to 5-FU (50 μmol/ml). The intracellular levels of the drug were determined using a primary antibody against 5-FU followed by FITC-conjugated secondary antibody. Fluorescence images showed higher fluorescence staining of intracellular 5-FU in Capan-1 cells pre-treated with benzyl-α-GalNAc followed by 1 h of exposure to 5-FU, when compared to cells treated with 5-FU alone (Fig. 3A). We also observed an intense cytoplasmic staining surrounding the nucleus in Capan-1 cells pre-treated with the inhibitor (Fig. 3A, inset) when compared to the untreated controls. The fluorescence images observed for Capan-1 cells following 4 h of exposure to 5-FU (Fig. 3B) showed no qualitative difference in antibody uptake between cells pre-treated with *O*-glycosylation inhibitor when compared to controls. The pre-treatment of U-87 MG cells with benzyl-α-GalNAc did not alter the staining patterns of 5-FU following 1 or 4 h of drug exposure (Fig. 4A and B).

Table 1 shows the fluorescence intensities corresponding to the levels of 5-FU in Capan-1 and U-87 MG cells following 1 and 4 h of drug exposure. The quantitative data analyses were determined by a fully automated fluorescence microplate reader (see Section 2). The values observed for Capan-1 cells following 1 h of exposure to 5-FU were significantly higher when cells were pre-treated with benzyl-α-GalNAc

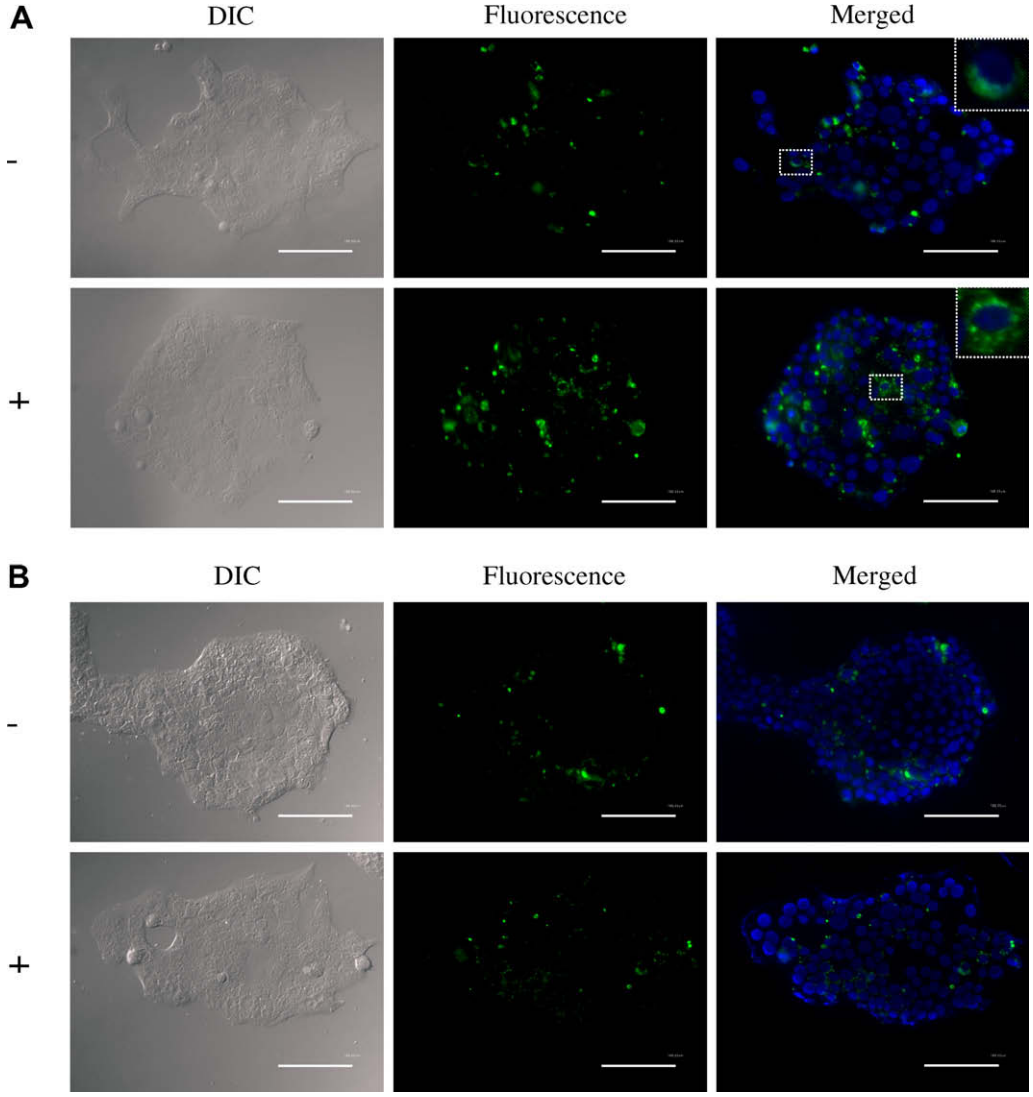

Fig. 3 – Effects of inhibiting O-glycosylation on 5-FU uptake by Capan-1 cells. Approximately 2×10^4 cells/ml were exposed to benzyl-α-GalNAc for 48 h followed by (A) 1 h or (B) 4 h exposure to 5-FU. Intracellular uptake of 5-FU was determined by immunofluorescence staining using anti-5-FU antibody. (A) Following 1 h of 5-FU exposure, higher staining of cells was observed when exposed to benzyl-α-GalNAc (+) as compared to cells not exposed to benzyl-α-GalNAc (–). The insets show cytoplasmic localisation of 5-FU and higher accumulation observed around the nucleus (blue) in cells exposed to benzyl-α-GalNAc (+) compared to control (–) cells. (B) The 5-FU staining following 4 h of drug exposure was not altered in the presence (+) or absence (–) of benzyl-α-GalNAc (20× magnification; bar: 100 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

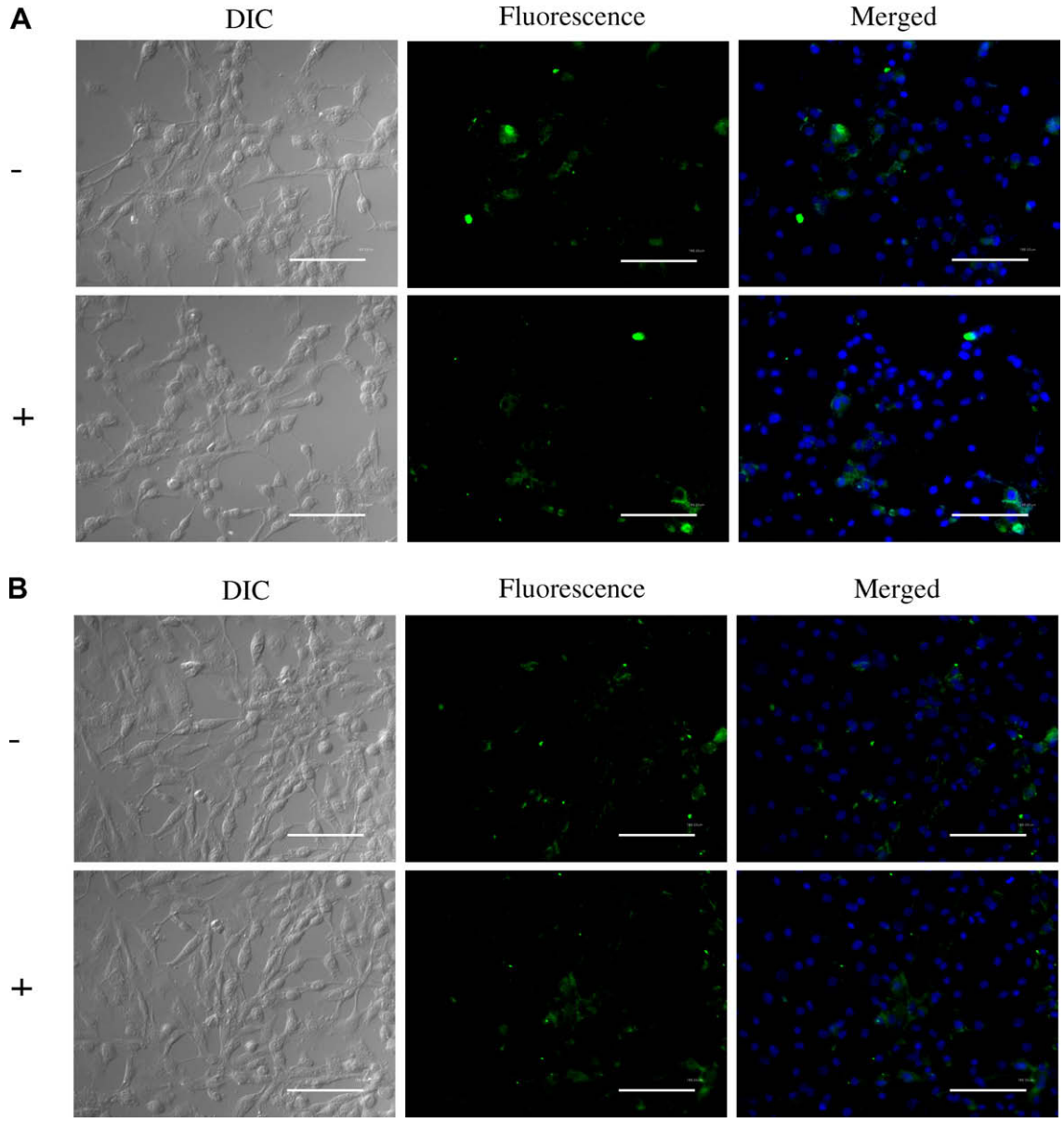

Fig. 4 – Effects of inhibiting O-glycosylation on 5-FU uptake by U-87 MG cells. Approximately 2×10^4 cells/ml were exposed to benzyl-α-GalNAc for 48 h followed by (A) 1 h or (B) 4 h exposure to 5-FU. Intracellular uptake of 5-FU was determined by immunofluorescence staining using anti-5-FU antibody. The intracellular staining of cells following 1 h or 4 h of 5-FU exposure was not altered in the presence (+) or absence (–) of benzyl-α-GalNAc (20× magnification; bar: 100 μm).

Table 1 – Intracellular uptake of 5-fluorouracil.

5-FU exposure time	1 h		4 h	
Benzyl-α-Ga1NAc	–	+	–	+
Fluorescence intensities, arbitrary units				
Capan-1	1696.9 ± 693.7	7271.4 ± 3556.2*	4253.0 ± 3045.9	3933.3 ± 2080.5
U-87 MG	3757.0 ± 2457.1	2346.9 ± 1241.1	3704.9 ± 2262.9	2989.4 ± 1024.3

* $P \leqslant 0.05$.

compared to 5-FU alone (*$P \leqslant 0.05$), whereas the values for U-87 MG cells were similar in the absence and presence of the O-glycosylation inhibitor. Moreover, the fluorescence intensities observed for both cell lines following 4 h exposure to 5-FU were also similar in the absence and presence of benzyl-α-GalNAc. These results suggest that the uptake of 5-FU by

pancreatic cancer cells was limited by the overexpression of mucins on the cell surface. The most significant uptake of 5-FU by Capan-1 cells exposed to benzyl-α-GalNAc was observed following 1 h of drug exposure. The fluorescence values observed following 4 h of exposure were not significantly higher when compared to 1 h.

The conversion of 5-FU to its active metabolites is a rapid process. In these reports, 5-FU metabolites were detected in Ls174t colon cancer cells within 4 h of exposure to drug.[19,20] This would explain why no significant increase in 5-FU uptake following 4 h of drug exposure when compared to 1 h was observed. In the present study, some fraction of the drug was probably converted to active metabolites, and the antibody has no cross-reactivity to the various metabolites. Finally, following exposure of U-87 MG cells to benzyl-α-GalNAc, we observed no change in the intracellular levels of 5-FU, thus offering additional support for the role of mucin in cytotoxic drug therapy.

4. Discussion

Amongst the various types of human cancers, patients diagnosed with lung, breast, prostate, colon and pancreatic cancers have the highest mortality rates.[1] It is interesting to note that the abovementioned organs (relative to the other types) not only express the highest levels of mucin under normal conditions, but in the tumours associated with each organ type as well.[5] Mucins overexpressed on the membrane surface of cancer cells have been exploited for several developmental treatment approaches. For example, tumours overexpressing MUC1 have been a target of monoclonal antibody-based therapies such as radioimmunoconjugates.[21] Moreover, clinical trials for vaccine-based therapies have been designed to elicit an immune response against MUC1 mucin.[22]

Recent studies have focused on the barrier function of mucin components in the glycocalyx. In these studies, mucins oriented on the surface of the respiratory epithelium were shown to limit the access of adenoviral vectors.[23] Also, mucins secreted on breast cancer cells decrease the accessibility of herceptin (monoclonal antibody) to its target receptor.[24] The inhibition of mucin *O*-glycosylation using benzyl-α-GalNAc enhanced the cytotoxic effects of 5-FU *in vitro* against the growth of human pancreatic cancer (Capan-1 and HPAF-II), but not against mucin-deficient (U-87 MG) cell lines.[12] In the present study, we investigated the extent to which the dense mucin mesh influences the antitumour activity of 5-FU. The overexpression of mucins in various cancer types has been reported.[5] The immunohistochemical staining of pancreatic tumour sections confirmed the overexpression of MUC1 in pancreatic tumours compared to non-mucin expressing tumours. The MUC1 expression levels in the different tumours studied here were as follows: Capan-1 > HPAF-II > U-87 MG tumours (Fig. 1A). A number of synthetic compounds have been used to inhibit glycosylation.[25] In the present study, we showed that benzyl-α-GalNAc inhibits mucin *O*-glycosylation in pancreatic tumours when the reagent was administered via intratumoural route. Immunohistochemistry showed that the intratumoural injections of benzyl-α-GalNAc significantly reduced the production of *O*-glycosylated chains (Fig. 1B-d).

When compared to many other conventional chemotherapeutic agents, 5-FU is administered at relatively high concen-

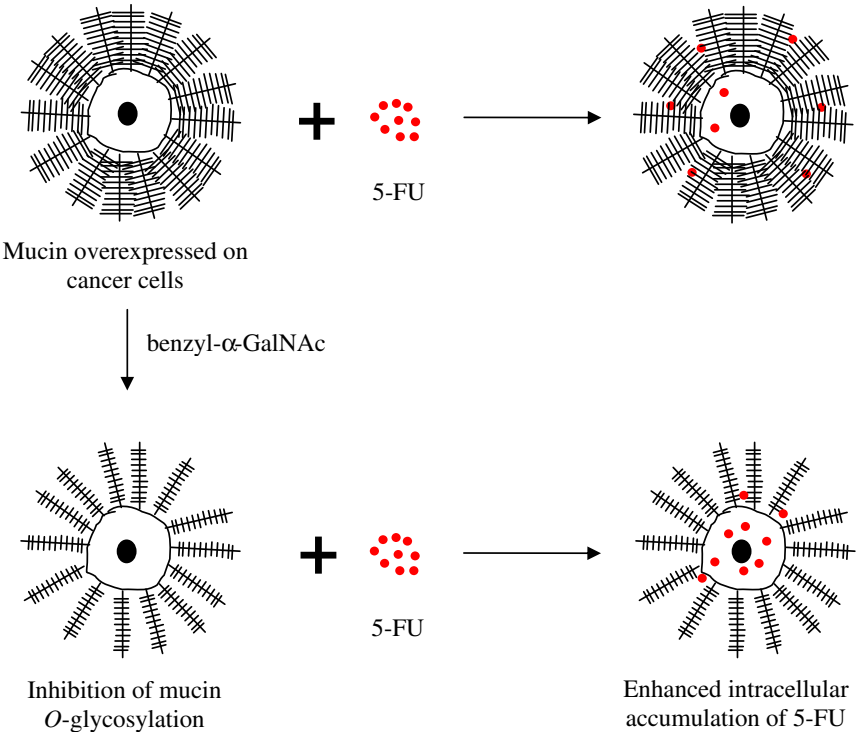

Fig. 5 – Schematic illustrates the enhanced intracellular accumulation of 5-FU following reduction of the mucin glycation mesh.

trations in order to achieve significant cytotoxic drug effects.[26] However, the susceptibility of pancreatic cancer cells to the drug can be enhanced by reducing the mucin glycation mesh.[12] We note that the concentration of inhibitor used herein, and previously, was non-toxic to cells *in vitro* and *in vivo*.

The contribution of mucins in the progression of pancreatic cancer has been discussed.[27] However, our fundamental understanding of the role of mucins in chemotherapy is limited. For example, cells that have acquired resistance to chemotherapeutic drugs showed altered expression levels of mucins.[28,29] In these studies, long-term exposure of HT-29 colon cancer cells to 5-FU and methotrexate resulted in the differentiation of cells to a relatively high mucin-secreting cellular phenotype. Interestingly, the level of *P*-glycoprotein (multidrug resistant protein) in these cells was not altered. Of note to this discussion, the upregulation of mucin expression in HT-29 cells in relation to intracellular drug uptake was not investigated in this report.

In the present study, we investigated the possible basic mechanism involved in the enhanced drug effects, following the reduction of the mucin glycation mesh. In order for 5-FU to exert its action a sufficient amount of drug must first reach the intracellular compartment, where 5-FU is converted to active metabolites.[17] The role of the mucus layer on the surface of normal epithelial cells in limiting the diffusion of nutrients and small molecules has been investigated.[30] The authors showed that small molecules such as ethanol diffuse slower through the mucin gel when compared to nutrients such as glucose. The role of mucins in limiting the efficiency of viral vectors for the transfection of epithelial cells has also been investigated.[23] These studies showed that mucins extending several hundred nanometres over the cell surface restrict the interactions between the viral vectors and cellular receptors.

There have been several reports discussing the influence of the mucus layer on absorption and diffusion of drugs across the gastrointestinal tract.[31,32] These studies investigated the permeability and diffusion of drugs through *in vitro* mucin producing cell culture models, such as a co-culture of Caco-2 and other mucin-secreting cells. In the present study, the intracellular uptake studies confirmed that the inhibition of mucin *O*-glycosylation in pancreatic cancer cells resulted in higher intracellular levels of 5-FU (Fig. 3A and Table 1). Furthermore, the exposure of the non-mucin expressing cell line to the inhibitor did not alter intracellular drug uptake (Fig. 4A and Table 1). These results suggest that the dense mucin mesh limits 5-FU uptake, given that a compromised mesh improved drug uptake (as illustrated in Fig. 5).

The factors influencing the diffusion of drug molecules across the mucus layer of epithelial cells had been reviewed previously.[33,34] Some factors are (1) size of the drug molecule, (2) charge of the drug molecule (which may influence the electrostatic interactions between the drug and the mucus components), (3) the hydrophilic–lipophilic character of the drug molecule and (4) the size of the mucus mesh. We speculate that some of these factors may also play a role in limiting the uptake of 5-FU by pancreatic cancer cells. The enhanced intracellular uptake of 5-FU correlates with the significant antitumour effects of 5-FU when applied in a reduced mucin environment (Fig. 2B). Since the drug is a relatively small cytotoxic drug molecule and is highly electronegative, 5-FU may get trapped within the dense mucin network for a longer residence time, which would delay, or even limit, direct contact with the cell membrane and entry into the cells.

On the basis of our experimental findings, we report herein that the overexpression of mucins in pancreatic cancer cells limits the intracellular uptake of 5-FU, thereby impeding its chemotherapeutic drug effect. Although the intratumoural concentration of benzyl-α-GalNAc used in this study was sufficient to demonstrate proof-of-concept, additional studies may be required to determine the dose that will achieve the maximum therapeutic benefit. Higher intratumoural concentrations of the inhibitor or prolonged tumour retention may produce even greater results.

In summary, compromising the formidable extracellular mucin barrier may create additional opportunities for pancreatic cancer treatment, and quite possibly for the treatment of other mucinous adenocarcinomas.

Conflict of interest statement

None declared.

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