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Gemcitabine loaded microbubbles for targeted chemo-sonodynamic therapy of pancreatic cancer

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ABSTRACT

Pancreatic cancer remains one of the most lethal forms of cancer with a 10-year survival of < 1%. With little improvement in survival rates observed in the past 40 years, there is a significant need for new treatments or more effective strategies to deliver existing treatments. The antimetabolite gemcitabine (Gem) is the most widely used form of chemotherapy for pancreatic cancer treatment, but is known to produce significant side effects when administered systemically. We have previously demonstrated the benefit of combined chemo-sonodynamic therapy (SDT), delivered using oxygen carrying microbubbles (O_2MB), as a targeted treatment for pancreatic cancer in a murine model of the disease. In this manuscript, we report the preparation of a biotin functionalised Gem ligand for attachment to O_2MBs (O_2MB-Gem). We demonstrate the effectiveness of chemo-sonodynamic therapy following ultrasound-targeted-microbubble-destruction (UTMD) of the O_2MB-Gem and a Rose Bengal loaded O_2MB (O_2MB-RB) as a targeted treatment for pancreatic cancer. Specifically, UTMD using the O_2MB-Gem and O_2MB-RB conjugates reduced the viability of MIA PaCa-2, PANC-1, BxPC3 and T110299 pancreatic cancer cells by > 60% ($p < 0.001$) and provided significant tumour growth delay (> 80%, $p < 0.001$) compared to untreated animals when human xenograft MIA PaCa-2 tumours were treated in SCID mice. The toxicity of the O_2MB-Gem conjugate was also determined in healthy non-tumour bearing MF1 mice and revealed no evidence of renal or hepatic damage. Therefore, the results presented in this manuscript suggest that chemo-sonodynamic therapy using the O_2MB-Gem and O_2MB-RB conjugates, is potentially an effective targeted and safe treatment modality for pancreatic cancer.

1. Introduction

The antimetabolite drug gemcitabine (Gem) is one of the most widely used chemotherapy for pancreatic cancer [1]. While surgical resection remains the only curative treatment for pancreatic cancer, it is only possible in ~20% of patients who initially present with the disease [2]. The remaining ~80% of patients are classified as non-resectable at the time of diagnosis with ~40% having metastatic disease and ~40% having borderline resectable or locally advanced pancreatic cancer (Borderline resectable pancreatic cancer (BRPC) or locally advanced pancreatic cancer (LAPC)), meaning that while the cancer is still localised to the pancreas, its size or anatomical proximity to major blood vessels makes it difficult for the surgeon to achieve a complete resection [2]. In a bid to improve resection rates, there has been a considerable emphasis in recent years on treating BRPC or LAPC patients with neoadjuvant chemo- or chemo-radiotherapy in an attempt to downstage tumours in advance of surgery [3]. Many of the drug-based treatments for pancreatic cancer involve Gem either as a stand-alone agent or in combination with other chemotherapies/radiotherapy [4]. However, while Gem has dominated the pancreatic cancer chemotherapy market for the past 20 years, the modest overall median survival of 5–7 months and significant off-target toxicity means alternative treatments or more targeted delivery methods would be highly beneficial [5].

Ultrasound targeted microbubble destruction (UTMD) is an emerging field in drug delivery and involves the use of low intensity ultrasound to disrupt microbubbles (MB) at a target site, releasing the

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attached payloads and encapsulated gas in a localised manner [6–8]. MBs are lipid or polymer stabilised gas filled particles approved for use as contrast agents in diagnostic ultrasound. At relatively low ultrasound pressures, MBs oscillate continuously, resulting in a strong ultrasound signal that enhances the quality of the diagnostic image. At higher acoustic pressures, inertially driven collapse of the MB leads to rupture and release of any encapsulated material at the target site [8]. An additional benefit of UTMD as a drug delivery strategy is that microbubble cavitation is known to enhance microscale mass transport through impermeable tissue and this has particular relevance when considering payload delivery to solid tumours [9]. Indeed, MB cavitation has been attributed to the improved uptake and efficacy of chemotherapy drugs in pre-clinical and clinical studies [10].

We have previously demonstrated the benefit of combined 5-fluorouracil (5-FU)/sonodynamic therapy (SDT) as a potential treatment for pancreatic cancer using oxygen-loaded lipid stabilised microbubbles (O_2MB) to deliver both the 5-FU antimetabolite and Rose Bengal SDT sensitiser to pancreatic tumours [11,12]. UTMD complements SDT as the ultrasound stimulus also enables activation of the otherwise non-active sensitiser, which in the presence of molecular oxygen, generates toxic quantities of reactive oxygen species (ROS) [13]. Given Gem has superseded 5-FU as the antimetabolite of choice for the treatment of pancreatic cancer, we have developed our MB platform to enable the delivery of Gem to facilitate combination treatment with SDT. Such a targeted combination therapy could find appeal as a neoadjuvant treatment to reduce tumour burden and ensure a curative R0 resection of the pancreas, or in the setting of unresectable disease, as a palliative therapy to provide improved tumour control with better quality of life.

To this end, we have synthesised a biotin functionalised Gem derivative (compound **4**, Fig. 1) and attached it to the surface of avidin functionalised O_2MB using the biotin-avidin interaction. The O_2MB-Gem conjugate was then used in combination with Rose Bengal functionalised O_2MB (O_2MB-RB) to facilitate combined chemo-sonodynamic therapy. The efficacy of chemo-sonodynamic therapy treatment using the MB conjugates was determined in vitro, in a panel of pancreatic cancer cells (BxPC3, MIA PaCa-2, PANC-1 and T110299) and in vivo in a MIA PaCa-2 subcutaneous xenograft murine model of the disease. To ensure the combined treatment was safe for potential translation to the clinic, we also conducted a safety study in healthy non-tumour bearing MF-1 mice, to establish the impact of treatment on key blood biochemical markers and liver/kidney histology.

2. Materials & methods

2.1. Reagents and materials

1,2-dibehenoyl-*sn*-glycero-3-phosphocholine (DBPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG(2000)) and DSPE-PEG(2000)-biotin were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Oxygen gas was purchased from BOC Industrial Gases UK and perfluorobutane (PFB) was purchased from Apollo Scientific Ltd. Phosphate Buffered Saline (PBS) was purchased from Gibco, Life Technologies, UK. Glycerol and propylene glycol (1 kg, hydrolysed) were purchased from Sigma Aldrich (UK). Optical microscope images were obtained using a Leica DM500 optical microscope. Rose Bengal sodium salt, NHS-biotin, gemcitabine, MTT assay kit, avidin, chloroacetic acid, 4-dimethylaminopyridine (DMAP), hydroxybenzotriazole (HOBt), *N,N′*-dicyclohexylcarbodiimide (DCC), anhydrous dimethylformamide (DMF), and ethanol were purchased from Sigma Aldrich (UK) at the highest grade possible. Biotin, di(*N*-succinimidyl) carbonate and 2-aminoethanol were purchased from Tokyo Chemical Industry UK Ltd.

2.2. Synthesis of biotin-gem conjugate (4)

(2R,3R,5R)-5-(4-amino-2-oxopyrimidin-1(2H)-yl)-4,4-difluoro-3 hydroxytetrahydrofuran-2-yl)methyl (2-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)ethyl) carbonate): The protocol for the preparation of **4** is shown in Fig. 1. The synthesis of **2** has previously been described [13]. To a dichloromethane (DCM) (10 mL) solution of **2** (0.28 g, 0.9 mmol), 4-nitrophenyl chloroformate (0.59 g, 2.9 mmol), diisopropylethylamine (DIPEA) (0.50 g, 3.9 mmol) and a catalytic

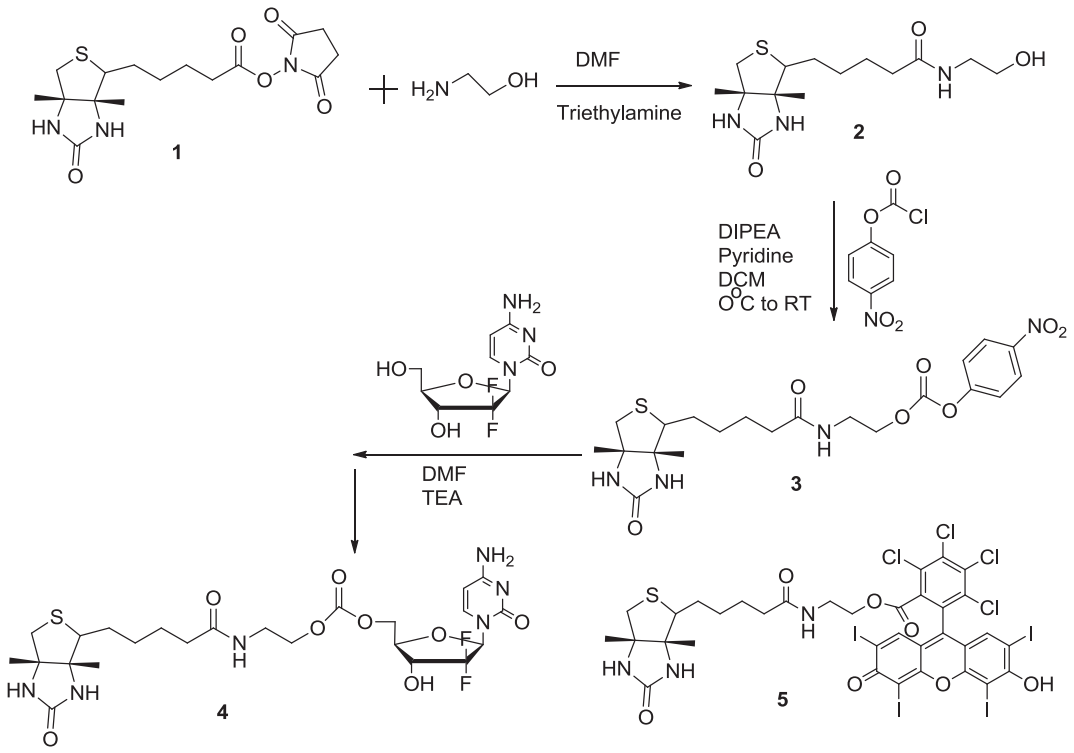

Fig. 1. Synthetic scheme for the preparation of Biotin-Gem (**4**) with the structure of biotin-Rose Bengal (**5**) also shown.