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Peptide-functionalized nanoparticles for selective targeting of pancreatic tumor

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ABSTRACT

Chemotherapy for pancreatic cancer is hampered by the tumor's physio-pathological complexity. Here we show a targeted nanomedicine using a new ligand, the CKAAKN peptide, which had been identified by phage display, as an efficient homing device within the pancreatic pathological microenvironment. Taking advantage of the squalenoylation platform, the CKAAKN peptide was conjugated to squalene (SQCKAAKN) and then co-nanoprecipitated with the squalenoyl prodrug of gemcitabine (SQdFdC) giving near monodisperse nanoparticles (NPs) for safe intravenous injection. By interacting with a novel target pathway, the Wnt-2, the CKAAKN functionalization enabled nanoparticles: (i) to specifically interact with both tumor cells and angiogenic vessels and (ii) to simultaneously promote pericyte coverage, thus leading to the normalization of the vasculature likely improving the tumor accessibility for therapy. All together, this approach represents a unique targeted nanoparticle design with remarkable selectivity towards pancreatic cancer and multiple mechanisms of action.

1. Introduction

Pancreatic cancer is a devastating disease which represents the fourth leading cause of cancer-related deaths in the European Community and in North America [1]. The median survival is less than 6 months and the maximum is 5 years for 6% of patients [2]. Despite this high mortality, pancreatic cancer represents only the 10th most common cause of new cancers. This imbalance between frequency and mortality is because most patients are diagnosed at an unresectable, advanced and metastatic stage for which, at the moment, only palliative treatments are available, due to the lack of sensitivity of pancreatic cancer to many chemotherapeutic drugs [2]. Despite the decline over the past decade in the global mortality related to lung, colon, prostate and breast cancer, the progresses in pancreatic cancer therapy have remained exceedingly slow and disappointing without impairing the death rate. Since 1996, gemcitabine (dFdC), a nucleoside analogue that blocks DNA replication, has been the major chemotherapeutic agent for pancreatic cancer treatment [2]. Despite its weak response rate of 5% and the modest overall survival benefit, this drug still remains the first-line treatment in clinical practice.

Several combined protocol therapies with gemcitabine have been tested in randomized clinical trials [3,4]. Only erlotinib, an inhibitor of the epidermal growth factor receptor and recently (September 2013) Abraxane (paclitaxel albumin-bound nanoparticles) have been approved by the FDA in combination with gemcitabine due to significant improvement in patient survival and delay in tumor growth [5–7]. Due to its rather hydrophilic character, gemcitabine is unable to passively diffuse across the plasma membrane but it is transported into the cell by either human equilibrative (hENT) or sodium-gradient nucleoside transporters. The resistance to gemcitabine treatment often arises from the down regulation of these nucleoside transporters which impairs gemcitabine membrane transport, as shown in both the pre-clinical experimental models and the clinic [8]. Gemcitabine is also rapidly inactivated into the blood, due to the fast metabolization by blood deaminases, into the inactive difluorouracil. Thus, a frequent administration schedule at a high drug dose (usually 800–1000 mg/m^2, 30 min infusion) is required. Additionally, the poor tumor tissue perfusion resulting from deprived vascularization, the formation of a dense stroma and the important heterogeneity of pancreatic cancer cells dramatically hamper drug efficacy and bioavailability in the tumor tissue, also leading to significant side effects [9].

Drug nanocarriers decorated with hydrophilic and flexible polymers, such as poly(ethylene glycol) (PEG), have been proposed to improve drug accumulation at the tumor target site through the so-called Enhanced Permeability and Retention (EPR) effect that takes advantage of the highly irregular tumor vasculature with abnormal heterogeneous density, the large pores on the endothelial walls and the reduced

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lymphatic drainage in tumor tissue [10,11]. However in several tumors, such passive targeting is inefficient. Therefore, the development of targeted therapeutic approaches, using nanoparticles (NPs) able to specifically bind to receptors mainly expressed onto malignant cells and relatively down regulated in healthy ones [12], represents an attractive therapeutic alternative [13–15]. To date, there are very few examples of drug-loaded nanoparticles functionalized with specific ligands showing a therapeutic efficacy in experimental pancreatic cancer models. For instance, nanocarriers were decorated with the epidermal growth factor (EGF) [16], the arginine-glycine-aspartic acid (RGD) peptide [17] or an antibody towards the transferrin receptor [18]. However, apart from the fact that these nanodevices exhibited poor drug loading [17,18] they were functionalized with homing devices not specific for pancreatic cancer since typically expressed also on several other types of healthy and/or cancer cells. This explains the inability of these materials to reach the pancreatic cancer tissue, even at a low concentration [19]. Therefore, the discovery of more specific ligands for pancreatic tumor targeting is urgently needed and represents an important challenge.

In this context, we have taken advantage of previous findings that, starting from a phage-displayed peptide library screening on mouse models of cancer, described peptides that selectively bind tumor vasculature [20]. The main advantage of this approach consists in the identification of novel peptide ligands, able to specifically interact with the target disease marker proteins, which are accessible *via* the systemic circulation within a definite pathological microenvironment. In particular Joyce et al. [20] identified a linear peptide composed by 6 amino acids (*i.e.* CKAAKN) which specifically bound tumor vessels in the RIP-Tag2 transgenic mice, a prototypical mouse model of multistage pancreatic islet cell carcinoma [20,21].

Motivated by these findings, we have constructed a novel efficient targeted nanomedicine for pancreatic cancer treatment, using squalene, a natural and biocompatible lipid as carrier material [21], gemcitabine as anticancer drug and the CKAAKN peptide as homing device. After chemical modification, the squalene was conjugated to either gemcitabine (SQdFdC) or to CKAAKN peptide (SQCKAAKN).

2. Materials and methods

2.1. Materials

1,1′,2-Trisnor-squalenic aldehyde was obtained from squalene as previously described [21]. Gemcitabine hydrochloride (difluorodeoxycytidine (dFdC)) was purchased from Sequoia Research Products Ltd (UK). 4-(*N*)-trisnorsqualenoyl-gemcitabine (SQdFdC) was obtained as previously reported [22]. CKAAKN peptide was purchased from CASLO Laboratory Aps (Denmark). Squalene, dextrose and all other reagents were obtained from Sigma-Aldrich Chemical (Italy). All solvents were of analytical grade from Carlo Erba Reagenti (Italy) or VWR (France). The ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance 300 (300 MHz, 75 MHz) or Bruker Avance 400 (400 MHz, 100 MHz) spectrometers in $CDCl_3$, CD_3COCD_3, CD_3OD or D_2O. Multiplicities of resonances are described as broad (b), singlet (s), doublet (d), triplet (t), or multiplet (m). Recognition of methyl, methylene, methine, and quaternary carbon nuclei in ^{13}C NMR spectra rests on the J-modulated spin-echo sequence. Mass spectra were obtained using electrospray ionization (ESI) conditions in a positive-ion or a negative-ion mode either on Finnigan-MAT TSQ 700 spectrometer (CA) or Esquire LC Bruker spectrometers. All reactions involving air- or water-sensitive compounds were routinely conducted in glassware which was flame-dried under a positive pressure of nitrogen. The reactions were monitored by thin-layer chromatography (TLC) on F254 silica gel pre-coated plates. After development, the plates were viewed under UV light (254 nm) and visualized with I_2 or Kägi–Misher reagents. Flash-column chromatography was performed on CombiFlash® Rf systems (Teledyne ISCO, Italy) on appropriate columns (silica or RP18). All solvents were distilled prior to flash chromatography.

2.2. Synthesis and characterization of 6-(maleimidyl)-hexanoic acid (trisnor-squalenylidene)-hydrazide (9)

A solution of 1,1′,2-trisnor-squalenic aldehyde **(8)** (0.334 g, 0.868 mmol) in CH_2Cl_2 was added to dry methanol (15 mL). The resulting mixture was sonicated for a few minutes until complete dissolution. [6-(maleimido)hexanamido]azanium trifluoroacetate **(7)** [23] (0.306 g, 0.868 mmol) and 4 Å molecular sieves (200 mg) were then added and the reaction mixture was stirred for 1 h at room temperature under nitrogen. The formation of the desired product **(9)** was monitored by TLC (petroleum ether/ethyl acetate 1/1 v/v, R_f: 0.65). The mixture was filtered and concentrated under reduced pressure. The residue was taken into water (5 mL) and extracted with CH_2Cl_2 (3 × 15 mL). The combined organic phases were dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. Purification by flash-chromatography on silica column, eluting with a gradient of petroleum ether to petroleum ether/ethyl acetate 60/40 v/v, gave the product as a light yellow waxy material (0.211 g, 63% yield) (Supplementary material, Fig. S1).

^{1}H NMR ($CDCl_3$) δ: 8.39 (s, 1H, C*H*═NN), 7.05 (t, J = 5.2 Hz, 1H, N*H*CO), 6.68 (s, 2H, CO–*CH*═*CH*CO), 5.14–5.07 (m, 5H, *H*C═C(CH_3)), 3.54–3.49 (t, J = 7.2 Hz, 2H, *CH*$_2$N), 2.70–2.50 (m, 2H, *CH*$_2$CONH), 2.40–1.90 (m, 20H, ═C(CH_3)*CH*$_2$*CH*$_2$), 1.80 (s, 3H, HC═C(*CH*$_3$)$_2$), 1.76–1.65 (m, 12H, HC═C(*CH*$_3$)CH$_2$), 1.62–1.60 (m, 4H, NCH$_2$*CH*$_2$CH$_2$*CH*$_2$CH$_2$CON), 1.41–1.33 (m, 2H, NCH$_2$CH$_2$*CH*$_2$CH$_2$CH$_2$CON). ^{13}C NMR ($CDCl_3$) δ: 171.2, 166.3, 147.3, 135.8, 135.7–132.0, 125.9–124.7, 42.5, 39.7–26.4, 38.2, 36.6, 31.0, 26.9, 25.9, 24.6–16.4, 22.4. MS (EI): m/z(%) 81 (70), 110 (100), 192 (55), 591 (3). HPLC analysis: Symmetry C18 column, 5 μm (Merck, Italy) equipped with a C18 column guard, elution with 100% methanol, detection by UV adsorption measurement at 237 nm (flow rate 1 mL/min, t_r = 5.79 min). Peak heights were recorded and processed on a CBM-10A Shimadzu interface.

2.3. Synthesis and characterization of the Michael adduct of CKAAKN and 6-(maleimidyl)-hexanoic acid (trisnor-squalenylidene)-hydrazide (SQ-CKAAKN, **(5)**)

A mixture of **(9)** (13.5 mg, 0.0229 mmol) and CKAAKN peptide (**4**) (7.25 mg, 0.0114 mmol) in dimethylformamide DMF/H_2O 3/1 v/v (2 mL) was stirred for 3 h at 40 °C. The reaction mixture was then concentrated under reduced pressure and the crude product was taken into diethyl ether to remove unreacted maleimide. The supernatant was withdrawn after decantation (3 times). In order to eliminate unreacted peptide, the solid was dissolved in methanol and filtered through a sintered glass funnel. The product was obtained as a translucent waxy material (10 mg, 0.0082 mmol, 70% yield); MS (+ESI): m/z (%) = 1226.0 (100) $[MH]^+$, 614.5 (2) (Fig. 1).

2.4. Preparation of nanoparticles

SQdFdC and SQdFdC/SQCKAAKN nanoparticles were prepared by the nanoprecipitation technique [24]. Practically, for SQdFdC NPs, SQdFdC was dissolved in ethanol (40 mg/mL) and then added dropwise under magnetic stirring into 1 mL of MilliQ® water (ethanol/water 0.1/1 v/v). Formation of the nanoparticles occurred spontaneously without using any surfactant. After solvent evaporation under reduced pressure, an aqueous suspension of pure SQdFdC nanoparticles was obtained (final SQdFdC concentration 4 mg/mL). For SQdFdC/SQCKAAKN NPs the two compounds were dissolved in 0.1 mL of ethanol at 1:0.01 SQdFdC/SQCKAAKN molar ratio. The organic solution was then added dropwise under magnetic stirring into 1 mL of MilliQ® water (ethanol/water 0.1/1 v/v). After solvent evaporation under reduced