Peptide-functionalized Nanoparticles for Selective Targeting of Pancreatic Tumor

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ABSTRACT SUMMARY
Chemotherapy of pancreatic cancer is hampered by its physio-pathological complexity. In this study, we present a novel targeted nanomedicine which employs as ligand a new peptide identified by phage display screening. Taking advantage from the squalenoylation concept,[1] this peptide was coupled to squalene (SQ) and the resulting bioconjugate was co-nanoprecipitated with the squalenoyl prodrug of gemcitabine (SQdFdC) giving sub-200-nm-sized, near monodisperse nanoparticles (NPs). Cytotoxicity and cell internalization studies clearly demonstrated that peptide functionalization enabled the specific targeting of pancreatic cancer cells while decreasing interactions with the healthy ones. In vivo studies in RIP-Tag2 mice, a model of spontaneously arising pancreatic cancer, demonstrated the higher efficacy of peptide-decorated NPs, which resulted from a dual activity on cancer and tumor vasculature cells.

Due to its specific targeting of the Wnt-2 signaling pathway, recently correlated to pancreatic tumorigenesis, this peptide may be considered as a novel efficient homing device within the pancreatic pathological microenvironment. To our knowledge, this approach is the first successful example of pancreatic cancer targeted nanomedicine with unique selectivity and multiple mechanism of action.

INTRODUCTION
Pancreatic cancer is a devastating disease which represents the fourth leading cause of cancer-related death in Europe and North America.[2] The median survival is less than 6 months and the maximum is 5 years for the 6% of patients, mainly because most patients are diagnosed at an unresectable, advanced and metastatic stage for which, at the moment, only palliative treatments are available. Since 1996, gemcitabine (dFdC) is the major chemotherapeutic agent for pancreatic cancer treatment. Despite the weak response rate and the modest overall survival benefit, this drug still remains the first-line treatment in the clinical practice. Combined therapies have been also tested but with no important clinical outcomes. The limited efficacy of gemcitabine is due to its rapid blood metabolization and the insurgence of resistance phenomena. In addition, the formation of a dense stroma, the limited tumor tissue vascularization and the heterogeneity of pancreatic cancer cells dramatically hamper drug efficacy and bioavailability in the tumor.

In this context, functionalized nanocarriers able to specifically target receptors overexpressed on cancer cells and relatively down regulated on healthy ones, represent an attractive therapeutic alternative. Currently, the discovery of novel specific ligands for pancreatic tumor targeting represents an important challenge. In this study, we have selected a peptide which specifically binds tumor vessels in the RIP-Tag2 transgenic mice.[3] Remarkably, this peptide was found capable of homing progenitor angiogenic cells as well as pancreatic stroma and cancer cells.

Motivated by these findings, we have constructed new targeted NPs for pancreatic cancer treatment by using squalene (SQ), a natural and biocompatible lipid, as carrier material, gemcitabine as anticancer drug and this new peptide as targeting agent.

EXPERIMENTAL METHODS

Synthesis of bioconjugates.
Squalenoyl derivatives were synthesized by conjugation of dFdC or the peptide to chemically-modified SQ. Briefly, a 6-maleimido-caproylhydrazide hydrophobic spacer was coupled to the 1,1′,2-trisnorqualenic aldehyde leading to formation of the squalene maleimide. Then, the SQPeptide bioconjugate was obtained by linkage of the peptide to the maleimide via a thio-ether bond. SQdFdC was prepared as previously described. [1]

Nanoparticle formulation and characterization.
SQdFdC and SQdFdC/SQPeptide nanoparticles were prepared by the nanoprecipitation technique. Cholesterolyl BODIPY FL C12 dye was used to prepare fluorescent NPs allowing tracking them in vitro. Size and polydispersity index of NPs were determined by dynamic light scattering and confirmed by CryoTEM microscopy. Surface charge of NPs was determined by measuring their electrophoretic mobility and expressed as their zeta potential. Targeting ability of the functionalized NPs was investigated by surface plasmon resonance (SPR).

Cell culture and in vitro studies.
Human pancreatic carcinoma cell lines MIA PaCa-2, BxPC3, PANC-1, breast cancer cell line MCF-7 and embryonic murine fibroblasts NIH/3T3 were obtained from ATCC and maintained as recommended.

Peptide receptor-negative and receptor-positive cells were identified by western blot (WB) and immunohistochemistry analysis.
The in vitro cytotoxicity of NPs was evaluated by MTT colorimetric assay. Cell internalization was investigated by flow cytometry and co-culture assays using fluorescent-labeled NPs.

**In vivo therapeutic efficacy.** Tumor-bearing RIP-Tag2 mice (12 weeks of age) were randomized and assigned to 4 groups which received four intravenous injections on days 0, 3, 7 and 11 with either (i) 15 mg/Kg dFdC; (ii) SQdFdC NPs at dFdC equivalent dose of 15 mg/kg; (iii) SQdFdC/SQPeP NPs at dFdC equivalent dose of 15 mg/kg or (iv) saline control solution. A pre-treatment with dexamethasone (5mg/kg) was performed by intramuscular injection 4h before the treatment. Mice were monitored regularly for changes in weight and health status and humanely sacrificed on day 12. Immunostaining of tissues was then performed to assess apoptosis index, vessel or pericyte coverage and peptide-receptor expression.

**RESULTS AND DISCUSSION**

The SQPeP bioconjugate was synthesized by conjugation of the thiol group of the N-terminal cysteine of the peptide to squalene, previously modified by the introduction of a lipophilic chain with a maleimide terminal group. Despite the difficulty to allow the hydrophilic peptide to react with the lipophilic squalene derivative, the Michael-type addition allowed to obtain the desired conjugate with a satisfactory 70% yield. Thanks to their similar squalenooy moiety, the dissolution of the two bioconjugates (i.e., SQdFdC and SQPeP) in ethanol and the simple addition of this ethanolic solution to water led to the spontaneous formation of NPs without requiring any surfactant. NPs showed high drug loading (40%) and a narrow size distribution (mean diameter of 130-170 nm).

SPR assay confirmed surface localization of the peptide and the ability to act as Wnt-2 ligand. An important signal was obtained with SQdFdC/SQPeP NPs, whereas no signal was observed under identical conditions with non-functionalized SQdFdC NPs (Fig. 1).

After 6 h of incubation with MIA Paca-2 cells, the capture of functionalized NPs was found 14-fold higher than the non-functionalised ones (Fig. 2c). Functionalized NPs were internalized by a receptor-mediated mechanism, allowing to achieve selective tumor cell uptake, while decreasing non-specific accumulation into healthy cells. The selective capture of functionalized NPs by cancer cells was further confirmed in a co-culture (NIH/3T3/MIA PaCa-2) experiment. Confocal images showed similar uptake of non-functionalized NPs in both tumor and normal cells (Fig. 2a), whereas functionalized NPs were selectively captured by tumor cells (Fig. 2b).

**CONCLUSION**

In a nutshell, a novel, efficient and easy to prepare nanomedicine for pancreatic cancer treatment has been presented. The superior efficacy of peptide-decorated nanoparticles clearly highlights the key role of the active targeting in the improvement of the therapeutic efficacy of gemcitabine in experimental pancreatic cancers.

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