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Targeting of p32 in peritoneal carcinomatosis with intraperitoneal linTT1 peptide-guided pro-apoptotic nanoparticles

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ABSTRACT

Gastrointestinal and gynecological malignancies disseminate in the peritoneal cavity - a condition known as peritoneal carcinomatosis (PC). Intraperitoneal (IP) administration can be used to improve therapeutic index of anticancer drugs used for PC treatment. Activity of IP anticancer drugs can be further potentiated by encapsulation in nanocarriers and/or affinity targeting with tumor-specific affinity ligands, such as tumor homing peptides. Here we evaluated a novel tumor penetrating peptide, linTT1 (AKRGARSTA), as a PC targeting ligand for nanoparticles. We first demonstrated that the primary homing receptor for linTT1, p32 (or gC1qR), is expressed on the cell surface of peritoneal carcinoma cell lines of gastric (MKN-45P), ovarian (SKOV-3), and colon (CT-26) origin, and that peritoneal tumors in mice and clinical peritoneal carcinoma explants express p32 protein accessible from the IP space. Iron oxide nanoworms (NWs) functionalized with the linTT1 peptide were taken up and routed to mitochondria in cultured PC cells. NWs functionalized with linTT1 peptide in tandem with a pro-apoptotic [$_D$(KLAKLAK)$_2$] peptide showed p32-dependent cytotoxicity in MKN-45P, SKOV-3, and CT-26 cells. Upon IP administration in mice bearing MKN-45P, SKOV-3, and CT-26 tumors, linTT1-functionalized NWs showed robust homing and penetration into malignant lesions, whereas only a background accumulation was seen in control tissues. In tumors, the linTT1-NW accumulation was seen predominantly in CD31-positive blood vessels, in LYVE-1-positive lymphatic structures, and in CD11b-positive tumor macrophages. Experimental therapy of mice bearing peritoneal MKN-45P xenografts and CT-26 syngeneic tumors with IP linTT1-$_D$(KLAKLAK)$_2$-NWs resulted in significant reduction of weight of peritoneal tumors and significant decrease in the number of metastatic tumor nodules, whereas treatment with untargeted $_D$(KLAKLAK)$_2$-NWs had no effect. Our data show that targeting of p32 with linTT1 tumor-penetrating peptide improves tumor selectivity and antitumor efficacy of IP pro-apoptotic NWs. P32-directed intraperitoneal targeting of other anticancer agents and nanoparticles using peptides and other affinity ligands may represent a general strategy to increase their therapeutic index.

1. Introduction

Gastrointestinal and gynecological malignancies often disseminate in the peritoneal space and trigger severe complications such as bowel obstruction and the formation of ascites. At the time of diagnosis, peritoneal metastases are present in about 50% of gastric, 30% of ovarian, and 40% of colorectal cancer patients [1]. This condition, known as peritoneal carcinomatosis (PC), has a poor prognosis with a median survival of only a few months [2]. PC is a vexing condition as complete surgical removal of disseminated microlesions is impossible and systemic chemotherapy has a limited anticancer effect due to poor vascularization of tumor nodules and the presence of peritoneum-plasma barrier [3,4]. Intraperitoneal (IP) chemotherapy is used to achieve an increased concentration of the anticancer drugs in the peritoneal cavity and to reduce systemic exposure [5]. IP therapy using mildly heated drug solutions, Hyperthermic IntraPeritoneal Chemotherapy (HIPEC), further enhances drug penetration into malignant tissue, and combining HIPEC with cytoreductive surgery results in

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improved efficacy of the treatment [6–9]. Encapsulation of cytotoxic drugs in nanoformulations has been also shown to improve the anticancer effect of IP chemotherapy [10]. Even with these improvements, many patients develop recurrent disease [11]. Ideally, PC treatment would not rely on specialized, complex equipment and procedures, and would instead be based on smart drugs that are potent, tumor selective, and show minimal peritoneal and systemic toxicities.

Tumor targeting with affinity ligands, such as peptides or antibodies, utilizes malignancy-associated molecular markers in the tumor microenvironment to deliver payloads to cancerous tissues [12,13]. Tumor homing peptides enable delivery of drugs and diagnostic compounds directly into tumors, thereby improving tumor detection and increasing the efficacy of the treatment while reducing side effects [14]. Tumor homing peptides identified by *in vivo* phage biopanning screens are particularly well suited for NP targeting, as phages used as scaffolds to display random peptides are biological nanoparticles themselves [15]. A series of recent studies have demonstrated the utility of iRGD, a tumor-penetrating peptide, for improved tumor-specific penetration of intraperitoneal drugs and nanoparticles and for enhanced IP chemotherapy in mice [16,17]. iRGD uses as recruitment receptors αv integrins, cell surface molecules commonly upregulated during angiogenic response and in tumor cells, and subsequently activates the tissue transport (CendR) pathway described below.

A recently identified tumor penetrating peptide TT1 (active both as a disulfide-bridged CKRGARSTC and as linTT1, AKRGARSTA) homes robustly to breast cancer in mouse models and enhances the antitumor potency of therapeutic payloads [18,19]. The primary homing receptor for TT1 family of peptides is p32 (also known as gC1qR), a mitochondrial protein aberrantly expressed on the cell surface of activated malignant and stromal cells in solid tumors, often in hypoxic areas deep in the tumor tissue [20]. TT1 belongs to a novel class of tumor targeting peptides, tumor penetrating C-end Rule (CendR) peptides characterized by a multistep homing and tumor penetration pathway. After binding to p32 TT1 peptide is proteolytically cleaved by a urokinase type plasminogen activator at the second arginine residue (AKRGA**R**STA) and the processed peptide acquires affinity towards tissue penetration receptor NRP-1 *via* its C-terminal RGAR CendR motif [19] to trigger vascular exit and tumor penetration [21,22].

Here, we set out to explore potential applications of linTT1 peptide as an IP targeting probe to PC lesions. As nanocarriers we used dextran-coated and PEGylated paramagnetic iron oxide nanoworms (NW) – a nanoscale agent extensively validated for peptide-mediated tumor targeting as a drug carrier and a MRI contrast agent [23–30]. Aspect ratio is known to influence performance of iron oxide nanoparticles in biological systems [29]. First, compared to spherical iron oxide nanoparticles, iron oxide nanoworms have extended circulation half-life. Second, the elongated NWs, with their larger surface area, present multiple targeting ligands that can cooperatively interact with cell surfaces, rendering the platform well-suited for affinity targeting. Finally, linearly aggregated IO cores in IONWs generate improved T2-relaxivity for improved MR imaging [29]. We used intraperitoneal linTT1-functionalized NWs carrying pro-apoptotic $_D$[KLAKLAK]$_2$ effector module [19,31] for experimental therapy on a panel of peritoneal tumors in mice. Our data indicate that linTT1 peptide functionalization greatly improves tumor selectivity of NWs and increases therapeutic efficacy of a pro-apoptotic nanosystem based on the NWs.

2. Materials and methods

2.1. Materials

($K_3[Fe(CN)_6]$), HCl, Nuclear Fast Red solution, Xylene substitute, MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), isopropanol, Triton-X and Tween-20 were purchased from Sigma-Aldrich, Germany. Phosphate-buffered saline (PBS) was purchased from Lonza (Belgium).

2.2. Peptides and NW preparation

Peptides were synthesized using Fmoc/t-Bu chemistry on a microwave assisted automated peptide synthesizer (Liberty, CEM Corporation, NC, USA). Peptides were purified by HPLC using 0.1% TFA in acetonitrile-water mixtures to 90%–95% purity and validated by Q-TOF mass spectral analysis. Fluorescent peptides were synthesized by using 5(6)-carboxyfluorescein (FAM) with 6-aminohexanoic acid spacer attached to the N-terminus of the peptide. The peptides were N-terminally amidated and had free C-termini.

The NWs were aminated by combining 0.25 ml of 28% aqueous ammonium hydroxide (#44939, Sigma-Aldrich, Germany) with 1 ml of NWs with an iron concentration of 1.3 mg/ml and stirring at room temperature for 24 h. Subsequently, the NWs were purified by dialysis against PBS using 20,000 MW cut-off dialysis cassettes (#66030, Thermo Fisher Scientific, USA) at 4 °C for 48 h (adopted from [30]).

NWs coated with peptides were prepared as previously described [29,30]. Briefly, aminated NWs were PEGylated with maleimide-5KPEG-NHS (JenKem Technology, TX, USA). Peptides were coupled to NWs through a thioether bond between the thiol group of a cysteine residue added to the N-terminus of the peptide and the maleimide on the functionalized particles. Dynamic Light Scattering (DLS; Zetasizer Nano ZS, Malvern Instruments, UK) was used to assess the polydispersity and size of NW preparations. Transmission electron microscopy (TEM, Tecnai 10, Philips, Netherlands) was used to image the nanoparticles.

2.3. Cell lines and experimental animals

MKN-45P human gastric cancer cells were isolated from parental MKN-45P cells [32]. SKOV-3 human ovarian carcinoma and CT-26 mouse colon carcinoma cell lines were purchased from ATCC (SKOV-3 ATCC HBT-7; CT-26 ATCC CLR-2638). The cells were cultivated in DMEM (Lonza, Belgium) containing 100 IU/ml of penicillin, streptomycin, and 10% of heat-inactivated fetal bovine serum (GE Healthcare, UK) in 5% CO_2 atmosphere. For animal experimentation athymic nude mice were purchased from HSD and Balb/c mice were purchased from Charles River. Animal experimentation protocols were approved by Estonian Ministry of Agriculture, Committee of Animal Experimentation (Project #42).

2.4. In vitro binding of nanoworms to recombinant p32 protein

Recombinant hexahistidine–tagged p32 was bacterially expressed and purified as described [18]. For cell-free binding assays, Ni-NTA magnetic agarose beads (Qiagen, Germany) in binding buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.05% NP40, 5 mM imidazole) were coated with p32 protein (at 15 μg of protein/10 μL beads). Fluorescently labeled NWs were incubated with the p32-coated beads in binding buffer containing 1% BSA at RT for 1 h. Incubation was followed by washes and elution with 400 mM imidazole containing binding buffer. The fluorescence of eluted samples was quantified using a fluorescence plate reader (FlexStation II, Molecular Devices, CA, USA).

2.5. In vitro cell viability assay

Cell viability was assessed by colorimetric assay based on reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide by NAD(P)H-dependent cellular oxidoreductase to insoluble purple formazan. Briefly, cells were seeded in 96-well plates (10,000 MKN-45P or SKOV-3 cells, and 5000 CT-26 cells) and grown in full medium at 37 °C. At 24 h different concentrations of NWs (3, 10, 30, 100, 300 μg/ml iron) were added to the wells. After 6 h the medium was replaced with a fresh medium, and 24 h after the NW addition, 10 μL of 5 mg/ml MTT reagent in PBS was added. Two h later the medium was removed, the blue formazan crystals were dissolved in